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(Saranel Salinas)
Saranel Salinas

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellant:	Lovenberg et al.	Atty. Docket:	ORT-1528
Serial No.:	09/993,159	Art Unit:	1632
Filed:	November 5, 2001	Examiner:	Michael C. Wilson
For:	Histamine Receptor H3 Modified Transgenic Mice	Confirmation No.:	8725

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APPELLANT'S BRIEF UNDER 37 C.F.R. § 41.37

Sir:

In response to the Notification of Non-Compliant Appeal Brief (Form PTOL-462) dated May 6, 2005, Appellant submits this revised brief in triplicate.

If any fees are due in connection with the filing of this brief, please charge any necessary fees to Deposit Account No. 10-0750.

I. Real Party in Interest

The real party in interest in this appeal is Ortho-McNeil Pharmaceutical, Inc., as reflected in the assignment from the inventors of rights pertaining to the above-captioned application officially recorded on February 26, 2002, at Reel 012651, Frames 0730 and 0747.

II. Related Appeals and Interferences

There are no other appeals or interferences known to Appellant, the undersigned legal representative of Appellant, or the above-identified assignee that would directly affect, be directly affected by, or have a bearing on the Board's decision in the present appeal.

III. Status of Claims

Claims 1-7 are on appeal. All claims are under final rejection.

IV. Status of Amendments

Although the box for item 3 on Form PTOL-462 was checked by the Examiner, no amendment was filed subsequent to the final rejection. Indeed, the Advisory Action dated October 1, 2004, addresses not any amendment but a request for reconsideration filed on August 18, 2004. As reflected at page 2 of the Form PTOL-462, the request for reconsideration presented arguments and, for the convenience of the Examiner, a listing of claims as presented in a previous response. Accordingly, Appellant's original brief was not defective in identifying the status of any amendments after final rejection, for there were none. Nonetheless, as requested by the Examiner, this revised brief now reflects that Appellants did file a request for reconsideration after final rejection.

V. Summary of Invention

In one general embodiment, the invention is directed to a transgenic mouse having somatic and germ cells comprising a disruption in an endogenous histamine H3 receptor gene. The disruption is generated by targeted replacement with a non-

functional histamine H3 receptor gene, such that the mouse has an insensitivity to amnesic effects of scopolamine as demonstrable in a passive avoidance test as compared to a wild-type histamine H3 receptor mouse. See specification, e.g., page 2, lines 7-15, and, for illustrative purposes, the examples at pages 11-18. In a preferred embodiment, the mouse is fertile and transmits the non-functional histamine H3 receptor gene to its offspring. See specification, e.g., page 8, lines 27-28. In another preferred embodiment of the mouse, the non-functional histamine H3 receptor gene has been introduced into an ancestor of the mouse at an embryonic stage by microinjection of embryonic stem cells into mouse blastocysts. See specification, e.g., page 14, line 24, through page 14, line 24.

Another general embodiment of the invention is directed to a cell isolated from a transgenic mouse as described above. See specification, e.g., page 2, lines 10-14.

The inventive transgenic mice and the cells derived therefrom serve as valuable tools that may be used to elucidate function of the histamine H3 receptor and to evaluate the therapeutic effects of drugs that modulate function or the expression of the H3 receptor equivalents in human cells. See specification, page 2, lines 10-14. For example, the mice may be used to: dissect the *in vivo* role of histamine H3 receptor signaling pathways (specification, page 2, lines 1-2); establish a nonhuman model for diseases involving histamine H3 receptor equivalents in the human (specification, page 4, lines 8-10); study the functional role of a drug target by studying the defects resulting from the disrupted gene in a whole animal

(specification, page 7, lines 26-27); and allow the definition of the function of histamine H3 receptor which is critical in deciding the types of modulators most suitable in therapies (specification, sentence bridging pages 7 and 8).

An additional general embodiment of the invention is directed to a method for producing a transgenic mouse whose somatic and germ cells comprise a disruption in an endogenous histamine H3 receptor gene, wherein the disruption is generated by targeted replacement with a non-functional histamine H3 receptor gene. The method comprises: (a) introducing a histamine H3 receptor gene targeting construct comprising a selectable marker into a mouse embryonic stem cell; (b) introducing the embryonic stem cell into mouse blastocysts; (c) transplanting the blastocysts into a recipient pseudopregnant mouse; (d) allowing the blastocysts to develop to term; (e) identifying a transgenic mouse whose genome comprises a disruption of the endogenous histamine H3 receptor gene in at least one allele; and (f) breeding the mouse of step (e) to obtain a transgenic mouse whose genome comprises a homozygous disruption of the endogenous histamine H3 receptor gene, wherein the disruption results in the mouse having an insensitivity to amnesic effects of scopolamine as demonstrable in a passive avoidance test as compared to wild-type histamine H3 receptor mice. See specification, e.g., paragraph bridging pages 5 and 6, and pages 11-18. In a preferred embodiment of the inventive method, the introducing in step (a) is by electroporation or microinjection. See specification, page 11, lines 11-12.

VI. Issues

One issue in the present appeal is whether claims 1-7 satisfy the utility requirement of 35 U.S.C. § 101.

A closely related issue is whether claims 1-7 satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph.

VII. Grouping of Claims

The utility and enablement of the claimed methods of making a transgenic mouse and cells derived from the transgenic mouse derive from the utility and enablement of the claimed transgenic mouse. Thus, solely for purposes of the issues addressed in the present appeal, the claims stand or fall together.

VIII. Argument

The inventive transgenic mouse does have utility and is supported by an enabling disclosure of how to make and use it. Contrary to the Examiner's contention at page 4 of the Advisory Action dated October 1, 2004, Appellant has specifically addressed the rejection under 35 U.S.C. § 101 as well as the rejection under 35 U.S.C. § 112, first paragraph. Both rejections were primarily grounded on the same arguments. In originally making both the utility and enablement rejections, the Examiner argued:

> The specification teaches H3-/- mice are resistant to the amnesic effect of scopolamine. . . . The specification does not teach how to use mice that are resistant to the amnesic effect of scopolamine. The art at the time of filing did not teach how to use such a mouse. . . . [W]hile the phenotype of the mouse is specific, the function of H3 receptors in the role of the amnesic effect of scopolamine is not. The insensitivity to scopolamine implies H3 receptors merely play a role in "passive avoidance." It remains unknown how H3 receptors function in the amnesic effect of scopolamine.

First Office Action, dated September 23, 2003, page 2 and pages 3-4.

Thus, the Examiner has grounded the alleged failure of the claimed transgenic mouse to meet the how-to-use prong of the enablement requirement on the alleged failure to meet the utility requirement. Compare, e.g., In re Brana, 51 F.3d 1560, 34 U.S.P.Q.2d 1436, 1439 (Fed. Cir. 1995) ("[o]bviously, if a claimed invention does not have utility, the specification cannot enable one to use it"). Because the grounds for both rejections are, for the most part, intertwined, Appellant's original brief clearly noted that it was addressing both rejections together for the sake of brevity. Since the Examiner is requiring each ground of rejection to be addressed under a separate heading, Appellant presents below substantially duplicative arguments under two headings.

As noted by the Examiner at page 2 of Form PTOL-462, the following arguments, which are found in the original brief, include new arguments and new citations to case law. Appellant notes that there is no rule prohibiting appeal briefs from supplementing previous arguments or case citations.

The following arguments also include a new item of evidence--the Harris article provided at Appendix II (publications cited in support of Appellant's arguments). Appellant respectfully requests entry and consideration of this article, which was not earlier presented because Appellant uncovered it after receiving the Advisory Action, in which the Examiner refused to give the Durant et al. and Perez-Garcia et al. publications weight. Since Appellant believes there is ample basis for reversing the

rejections absent the Harris article, the arguments below may be considered without reference to this supplemental evidence in the event its entry is refused.

A. Claims 1-7 Satisfy the Utility Requirement of 35 U.S.C. § 101

In evaluating whether the closely related utility and enablement requirements have been met in the present case, it must be kept in mind that Appellant is claiming not a therapeutic drug targeting a histamine H3 receptor gene nor even the receptor gene itself, but a transgenic mouse having a disruption in an endogenous histamine H3 receptor gene generated by targeted replacement with a non-functional histamine H3 receptor gene. In other words, Appellant is claiming an $H3^{-/-}$ knockout mouse. The Examiner has failed to meet his burden of showing that one of ordinary skill in the art would reasonably doubt the asserted utility of the claimed invention.

As apparent from the assertion of usefulness in the specification, the claimed mouse has a substantial and practical utility as a research tool like other knockout mice in general. For example, compare Masaki et al., "Targeted Disruption of Histamine H_1-Receptor Attenuates Regulatory Effects of Leptin on Feeding, Adiposity, and UCP Family in Mice," *Diabetes*, vol. 50, February 2001, 385-391 (submitted with Supplemental Information Disclosure Statement dated August 6, 2003). Moreover, as has been noted by the Examiner at pages 2 and 4 of the first Office Action, the Toyota et al. article, "Behavioral Characterization of Mice Lacking Histamine H_3 Receptors," *Molecular Pharmacology*, vol. 61, 2002, 389-397 (also submitted with the Supplemental Information Disclosure Statement), at p. 396, concludes that the claimed transgenic mouse "should

prove extremely important for elucidating the role of H_3 receptors in a variety of peripheral and CNS functions as well as pathophysiological states that are associated with altered histaminergic activity." Even though the Toyota et al. article is not prior art, it provides some probative evidence that researchers have recognized that the $H3^{-/-}$ knockout mouse has substantial real-world value as a research tool.

Nonetheless, the Examiner has reasoned that "[f]urther research does not have a specific or substantial utility" (second Office Action, dated May 18, 2004, p. 3). But, as the Federal Circuit has observed, "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development." In re Brana, 34 U.S.P.Q.2d at 1442.

If the Examiner's apparent rule--that use for research is not a patentable utility--were to be adopted, it would in effect knock out the patentability of not only knockout animals, but also similar research tools *per se*. Various types of research tools used in the pharmaceutical industry, however, have been recognized as having value in the industry. See Integra LifeSciences I Ltd. v. Merck KGaA, 66 U.S.P.Q.2d 1865 (Fed. Cir. 2003) (in reference to the Examiner's observation in the Advisory Action that the Integra case does not mention knockout mice, Appellant notes that the case is cited in support of the point that a research tool *in general* can have patentable utility).

Regarding the particular type of research tool at issue in this appeal, knockout mice have been recognized as having real-world utility. As noted by Harris in "Transgenic knockouts as part of high-throughput, evidence based target selection and

validation strategies," *Drug Discovery Today*, vol. 6, no. 12, 12 June 2001, 628-636 (copy attached at Appendix II), at p. 633, "[w]ithin the pharmaceutical industry, transgenic animals, especially gene knockouts, are proving to be invaluable sources of functional information and tools that can be used in studies at various other stages of the drug discovery process . . . ."

Accordingly, even if the receptor gene knocked out in the claimed mouse were to have had no biological function or role yet correlated with it at the time of the invention, the mouse would still have real-world value as a research tool. In any event, the receptor knocked out in the mouse at issue is not an orphan one, for it already has various biological functions or roles associated with it.

Although the instant specification, like the literature, reflects that *all* of the functions or roles of the H3 receptor in signaling pathways or biological mechanisms have yet to be fully elucidated or validated, the specification and prior art nonetheless describe some functions or roles of the receptor that are credible. As indicated at page 10 of the present specification, the phenotypic findings correlate with the role of the receptor in cholinergic pathways modulating memory function or cognitive processes. See also, WO 95/11894 to Durant et al., entitled "Histamine H_3-Receptor Antagonists and Therapeutic Uses Thereof", and Perez-Garcia et al., *Psychopharmacology*, vol. 142, 1999, 215-220 (both of which were cited in the Supplemental Information Disclosure Statement). For example, the Durant et al. publication notes that histamine H3 receptor antagonists have utility in treating dementia disorders, such as Alzheimer's

disease. These two publications provide evidence that even modulators of histamine H3 receptor activity have substantial and credible utility, and therefore support that the claimed $H3^{-/-}$ mouse has substantial and credible utility as a pharmaceutical research and development tool, e.g., to help screen or develop such histamine H3-modulating compounds.

In the Advisory Action, the Examiner refused to give the Durant et al. and Perez-Garcia et al. publications any weight as supporting the credibility of the practical utility of the claimed $H3^{-/-}$ mouse. But, contrary to the Examiner's assertion at page 3 of the Advisory Action, the Durant et al. publication does link the H3 receptor to Alzheimer's disease (see, e.g., page 1, lines 16-35, and paragraph spanning pages 7 and 8), even though it does not prove or validate the receptor's role in the disease. In reference to the Perez-Garcia et al. article, the Examiner complained that this publication "does not teach any ligands of H3 receptor that treat disease" (Advisory Action, p. 3). As best understood by Appellant, in dismissing these publications the Examiner is taking the position that a pharmaceutical compound modulating the H3 receptor must be shown as effective in treating Alzheimer's or another disease in humans, or at least the receptor must be validated as a target for Alzheimer's or another disease, before the claimed knockout mouse can have utility.

The Examiner overlooks, however, that "[t]he stage at which [a pharmaceutical] invention . . . becomes useful is well before it is ready to be administered to humans." In re Brana, 34 U.S.P.Q.2d at 1442. Similarly, the stage at which a receptor molecule

becomes useful is well before it has been rigorously confirmed (i.e., validated) as a therapeutic target. Likewise, the stage at which a knockout mouse becomes useful is well before all, if not any, of the functions and roles of the particular gene disrupted have been identified.

Furthermore, the fact that the H3 receptor has not been validated as a target actually bears out the practical need for, and therefore the utility, of the claimed mouse. As explained by Harris et al. (Appendix II), p. 633, "[t]he most significant impact of transgenics is currently in the exploratory phase, where gene knockouts are predominantly, but not exclusively, created to support target validation as part of a disease-to-target strategy . . . ." Just as FDA approval is not a prerequisite for finding a pharmaceutical compound useful within the meaning of the patent laws, In re Brana, 34 U.S.P.Q.2d at 1442, receptor target validation is not a prerequisite for finding a receptor knockout useful.

Although the specification does not validate the H3 receptor as a disease target, the specification reasonably correlates H3 receptor modulation to a specific condition--insensitivity to amnesic effects of scopolamine. Additionally, the specification supplies illustrative examples of particular research-tool applications of the claimed knockout mouse. For example, the specification describes how the transgenic mouse is used in passive avoidance tests to study the efficacy of experimental histamine H3 receptor antagonists or modulators (see pages 17-18). In another example, the mouse is used for studying the effects of histamine H3 receptor antagonists on sleep-wake states (see

page 16). Thus, the specification itself evidences the utility of the inventive knockout mouse.

As evident from the foregoing, a person of ordinary skill in the art would readily appreciate that the inventive mouse has a utility that is specific, substantial, and credible. Accordingly, the claims on appeal satisfy the utility requirement under Section 101.

B. Claims 1-7 Satisfy the Enablement Requirement of 35 U.S.C. § 112, ¶ 1

The claims likewise satisfy the utility prong of the enablement requirement under Section 112, first paragraph. In evaluating whether the closely related utility and enablement requirements have been met in the present case, it must be kept in mind that Appellant is claiming not a therapeutic drug targeting a histamine H3 receptor gene nor even the receptor gene itself, but a transgenic mouse having a disruption in an endogenous histamine H3 receptor gene generated by targeted replacement with a non-functional histamine H3 receptor gene. In other words, Appellant is claiming an $H3^{-/-}$ knockout mouse. The Examiner has failed to meet his burden of showing that one of ordinary skill in the art would reasonably doubt that one of ordinary skill in the art could use the claimed invention without undue experimentation.

As apparent from the assertion of usefulness in the specification, the claimed mouse has a substantial and practical utility as a research tool like other knockout mice in general. For example, compare Masaki et al., "Targeted Disruption of Histamine H_1-Receptor Attenuates Regulatory Effects of Leptin on Feeding, Adiposity, and UCP Family

in Mice," *Diabetes*, vol. 50, February 2001, 385-391 (submitted with Supplemental Information Disclosure Statement dated August 6, 2003). Moreover, as has been noted by the Examiner at pages 2 and 4 of the first Office Action, the Toyota et al. article, "Behavioral Characterization of Mice Lacking Histamine H_3 Receptors," *Molecular Pharmacology*, vol. 61, 2002, 389-397 (also submitted with the Supplemental Information Disclosure Statement), at p. 396, concludes that the claimed transgenic mouse "should prove extremely important for elucidating the role of H_3 receptors in a variety of peripheral and CNS functions as well as pathophysiological states that are associated with altered histaminergic activity." Even though the Toyota et al. article is not prior art, it provides some probative evidence that researchers have recognized that the $H3^{-/-}$ knockout mouse has substantial real-world value as a research tool.

Nonetheless, the Examiner has reasoned that "[f]urther research does not have a specific or substantial utility" (second Office Action, dated May 18, 2004, p. 3). But, as the Federal Circuit has observed, "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development." In re Brana, 34 U.S.P.Q.2d at 1442.

If the Examiner's apparent rule--that use for research is not a patentable utility--were to be adopted, it would in effect knock out the patentability of not only knockout animals, but also similar research tools *per se*. Various types of research tools used in the pharmaceutical industry, however, have been recognized as having value in the industry. See Integra LifeSciences I Ltd. v. Merck KGaA, 66 U.S.P.Q.2d 1865 (Fed.

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Accordingly, even if the receptor gene knocked out in the claimed mouse were to have had no biological function or role yet correlated with it at the time of the invention, the mouse would still have real-world value as a research tool. In any event, the receptor knocked out in the mouse at issue is not an orphan one, for it already has various biological functions or roles associated with it.

Although the instant specification, like the literature, reflects that *all* of the functions or roles of the H3 receptor in signaling pathways or biological mechanisms have yet to be fully elucidated or validated, the specification and prior art nonetheless describe some functions or roles of the receptor that are credible. As indicated at page 10 of the present specification, the phenotypic findings correlate with the role of the

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In the Advisory Action, the Examiner refused to give the Durant et al. and Perez-Garcia et al. publications any weight as supporting the credibility of the practical utility of the claimed $H3^{-/-}$ mouse. But, contrary to the Examiner's assertion at page 3 of the Advisory Action, the Durant et al. publication does link the H3 receptor to Alzheimer's disease (see, e.g., page 1, lines 16-35, and paragraph spanning pages 7 and 8), even though it does not prove or validate the receptor's role in the disease. In reference to the Perez-Garcia et al. article, the Examiner complained that this publication "does not teach any ligands of H3 receptor that treat disease" (Advisory Action, p. 3). As best understood by Appellant, in dismissing these publications the Examiner is taking the position that a pharmaceutical compound modulating the H3 receptor must be shown

as effective in treating Alzheimer's or another disease in humans, or at least the receptor must be validated as a target for Alzheimer's or another disease, before the claimed knockout mouse can have utility.

The Examiner overlooks, however, that "[t]he stage at which [a pharmaceutical] invention . . . becomes useful is well before it is ready to be administered to humans." In re Brana, 34 U.S.P.Q.2d at 1442. Similarly, the stage at which a receptor molecule becomes useful is well before it has been rigorously confirmed (i.e., validated) as a therapeutic target. Likewise, the stage at which a knockout mouse becomes useful is well before all, if not any, of the functions and roles of the particular gene disrupted have been identified.

Furthermore, the fact that the H3 receptor has not been validated as a target actually bears out the practical need for, and therefore the utility, of the claimed mouse. As explained by Harris et al. (Appendix II), p. 633, "[t]he most significant impact of transgenics is currently in the exploratory phase, where gene knockouts are predominantly, but not exclusively, created to support target validation as part of a disease-to-target strategy . . . ." Just as FDA approval is not a prerequisite for finding a pharmaceutical compound useful within the meaning of the patent laws, In re Brana, 34 U.S.P.Q.2d at 1442, receptor target validation is not a prerequisite for finding a receptor knockout useful.

Although the specification does not validate the H3 receptor as a disease target, the specification reasonably correlates H3 receptor modulation to a specific condition--

insensitivity to amnesic effects of scopolamine. Additionally, the specification supplies illustrative examples of particular research-tool applications of the claimed knockout mouse. For example, the specification describes how the transgenic mouse is used in passive avoidance tests to study the efficacy of experimental histamine H3 receptor antagonists or modulators (see pages 17-18). In another example, the mouse is used for studying the effects of histamine H3 receptor antagonists on sleep-wake states (see page 16). Thus, the specification itself evidences the utility of the inventive knockout mouse.

As evident from the foregoing, a person of ordinary skill in the art would readily appreciate that the inventive mouse has a utility that is specific, substantial, and credible. Moreover, the artisan would appreciate that the full scope of the claimed invention is supported by an enabling disclosure.

The claims do not encompass any disruption of any histamine H3 receptor gene, but specify that the disruption is generated by targeted replacement with a non-functional histamine H3 receptor gene that results in the mouse having an insensitivity to amnesic effects of scopolamine as demonstrable in a passive avoidance test as compared to wild-type histamine H3 receptor mice. The Examiner has failed to explain why, considering the examples, teachings and other guidance provided in the present disclosure, coupled with the knowledge in the art, a person of ordinary skill would have required more than routine experimentation to identify viable disruptions and therefore to make and use mice within the scope of the claims. Since the Examiner has failed to

meet his burden of establishing a *prima facie* case of non-enablement with respect to the present claims, Appellant need not limit the claims to a particular embodiment illustrated in the specification. The claimed invention has specific, substantial, and credible utility, and is fully supported by an enabling disclosure.

IX. Conclusion

For the foregoing reasons, the final rejections of claims 1-7 under 35 U.S.C. § 101 and § 112, first paragraph, are in error and should be reversed.

Respectfully submitted,

Date: May 31, 2005

Linda S. Evans
Reg. No. 33,873
(858) 320-3406

Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, New Jersey 08933-7003

APPENDIX I

Claims on appeal:

1. A transgenic mouse whose somatic and germ cells comprise a disruption in an endogenous histamine H3 receptor gene, wherein said disruption is generated by targeted replacement with a non-functional histamine H3 receptor gene, and wherein said disruption results in said mouse having an insensitivity to amnesic effects of scopolamine as demonstrable in a passive avoidance test as compared to wild-type histamine H3 receptor mice.

2. The mouse of claim 1, wherein said mouse is fertile and transmits the non-functional histamine H3 receptor gene to its offspring.

3. The mouse of claim 1, wherein the non-functional histamine H3 receptor gene has been introduced into an ancestor of the mouse at an embryonic stage by microinjection of embryonic stem cells into mouse blastocysts.

4. The mouse of claim 1, wherein the non-functional histamine H3 receptor gene has been introduced at an embryonic stage by microinjection of embryonic stem cells into a mouse blastocyst.

5. A method for producing a transgenic mouse whose somatic and germ cells comprise a disruption in an endogenous histamine H3 receptor gene, wherein said disruption is generated by targeted replacement with a non-functional histamine H3 receptor gene, said method comprising:

   a) introducing a histamine H3 receptor gene targeting construct comprising a selectable marker into a mouse embryonic stem cell;
   b) introducing the embryonic stem cell into mouse blastocysts;
   c) transplanting the blastocysts into a recipient pseudopregnant mouse;
   d) allowing the blastocysts to develop to term;

e) identifying a transgenic mouse whose genome comprises a disruption of the endogenous histamine H3 receptor gene in at least one allele; and

f) breeding the mouse of step (e) to obtain a transgenic mouse whose genome comprises a homozygous disruption of the endogenous histamine H3 receptor gene, wherein said disruption results in said mouse having an insensitivity to amnesic effects of scopolamine as demonstrable in a passive avoidance test as compared to wild-type histamine H3 receptor mice.

6. The method of claim 5 wherein the introducing of step (a) is by electroporation or microinjection.

7. A cell isolated from the transgenic mouse of claim 1.

APPENDIX II

Publications in support of arguments:

XP-002238430

Targeted Disruption of Histamine H_1-Receptor Attenuates Regulatory Effects of Leptin on Feeding, Adiposity, and UCP Family in Mice

Takayuki Masaki, Hironobu Yoshimatsu, Seiichi Chiba, Takeshi Watanabe, and Toshiie Sakata

P.D. 01-02-2001
P. 385-391
7

Histamine neurons are widely distributed in the brain and suppress food intake through the histamine H_1 receptor (H_1-R) in the hypothalamus. To examine the role of neuronal histamine in leptin signaling pathways, we investigated the effects of H_1-R knockout (H1KO) mice on both food intake and mRNA expressions of uncoupling proteins (*UCP*s) as regulated by leptin, and concomitantly on basal changes in both expression of hypothalamic neuropeptides and diet-induced fat deposition in adipose tissues. H1KO mice showed no change in daily food intake, growth curve, body weight, or adiposity. Reflecting no specificity in these parameters, H1KO mice induced no basal changes in mRNA expression of hypothalamic neuropeptides, *ob* gene, or peripheral *UCP*s. Loading H1KO mice with a high-fat diet accelerated fat deposition and *ob* gene expression compared with the controls. Leptin-induced feeding suppression was partially attenuated in H1KO mice, indicating involvement of histamine neurons in feeding regulation as a downstream signal of leptin. Upregulation of fat *UCP* mRNA and reduction of body fat induced by central infusion of leptin were attenuated in the H1KO mice. These results show that H1KO mice are a novel leptin-resistant model and that H_1-R is a key receptor for downstream signaling of leptin in the brain that contributes to regulation of feeding, fat deposition, and *UCP* mRNA expression. *Diabetes* 50:385–391, 2001

Histamine neurons originating from the tuberomammillary nucleus of the posterior hypothalamus project diffusely in the brain to regulate energy homeostasis (1,2). Neuronal histamine has been shown to suppress food intake through histamine H_1-receptors (H_1-Rs) in the ventromedial hypothalamus (VMH) and the paraventricular nucleus (PVN) (3,4). It also alters thermoregulation (5). Energy deficiency in the brain, i.e., neural glucoprivation, activates histamine neurons in the hypothalamus (6) and augments glycogenolysis in the brain (7). Histamine neurons stimulate the sympathetic nervous system to increase lipolysis in the adipose tissue (8,9).

Leptin, an *ob* gene product (10), has been recently demonstrated to promote histamine turnover by affecting the post transcriptional process of histidine decarboxylase formation or histamine release per se (11). In addition, concentration or turnover rate of hypothalamic histamine was lowered in leptin-deficient *ob/ob* and leptin receptor–mutated *db/db* mice but it was increased in diet-induced obese animals (11). Leptin regulates metabolic efficiency and exerts anorectic action (12–14) through its hypothalamic long-form receptors, in the VMH, the dorsomedial hypothalamus, the arcuate nucleus and the ventral premammillary nucleus (15–17). The VMH, the PVN, and the arcuate nucleus are known as controlling centers of appetite and receive projections from histamine neurons (3,4,18,19).

From the viewpoint of energy metabolism, the uncoupling protein (UCP) family plays an essential role in energy homeostasis (20–22). Gene expression of these proteins is regulated by humoral and neuronal factors (23–28). Central administration of leptin upregulated gene expression of the *UCP* family (28). These findings suggest that signal transduction between leptin and histamine neurons may be involved in central regulation of the UCP family.

A growing body of rapidly advancing information on functional roles of histamine neurons together with their central signaling pathways is consistent with a concept that hypothalamic histamine neurons are very likely to contribute to central regulation of energy balance governed by leptin. To address this issue, we hypothesized that knockout of H_1-R (H1KO) might disrupt leptin signaling messages ranging from expression of hypothalamic neuropeptides to that of the *UCP* family and *ob* gene. A goal of the present study was to examine the essential roles of H_1-R in regulation of food intake and *UCP* expression.

RESEARCH DESIGN AND METHODS

Animals. Mature male C57BL/6J mice (Seac Yoshitomi, Fukuoka, Japan) and H1KO mice (Kyushu University, Fukuoka, Japan), at 0–30 weeks of age, were housed in a room illuminated daily from 0700 to 1900 (a 12:12 h light-dark cycle) at a temperature at 21 ± 1°C and humidity at 55 ± 5%. The mice were allowed access to standard powdered mouse food (CLEA Japan, Tokyo, Japan) and tap water ad libitum. Daily food consumption and body weight of the mice were measured at 0800. The measurement was monitored at least 7 days before each experiment. The animals used were treated in accordance with the Oita Medical University Guidelines for the Care and Use of Laboratory Animals.

Production and supply of H1KO mice. Male and female H1KO mice were maintained for backcrossing at Medical Institute of Bioregulation (Kyushu Uni

From the Department of Internal Medicine (T.M., H.Y., S.C., T.S.), School of Medicine, Oita Medical University, Oita; and the Department of Molecular Immunology (T.W.), Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan.

Address correspondence and reprint requests to Toshiie Sakata, Department of Internal Medicine, School of Medicine, Oita Medical University, Hasama, Oita, 879-5593, Japan. E-mail: sakata@oita-med.ac.jp.

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BAT, brown adipose tissue; H_1-R, H_1-receptor; HFD, high-fat diet; LFD, low-fat diet; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; POMC, proopiomelanocortin; PVN, paraventricular nucleus; UCP, uncoupling protein; VMH, ventromedial hypothalamus; WAT, white adipose tissue, WT, wild-type.

versity). The methods used to produce these mice are reported in detail elsewhere (29). Backcrossing H_1R-/- homozygous mice to the C57BL/6J strain for five generations resulted in the incipient congenial N4 mice of three genotypes (H_1R-/-, H_1R+/-, H_1R+/+) used here. All genotypes were confirmed using Southern blotting. In total, 178 offspring of heterozygous males and females were analyzed for the genotype at the age of 28 days. The following distribution was observed: +/+, n = 42; +/-, n = 90; -/-, n = 46. These results closely appropriate the expected ratio of 1:2:1, indicating that H_1-R deficiency does not adversely affect pre- or postnatal viability.

Measurement of food consumption and growth curve. The growth rate of the male H1KO and wild-type (WT) mice (6 mice/group) was monitored from their weaning at 1 week of age up to a 30-week period. Their daily food intake over 24 h was measured at 12 and 30 weeks of age. These mice were housed alone throughout the 30-week monitoring period under the acclimatized ambient condition described above.

Measurement of body composition and blood sampling procedures. Body weight, total fat weight, and percent fat were measured to detect the difference in body fat accumulation between male H1KO and WT mice at 12 and 30 weeks of age using 6 subjects for each. Total body weight, fat weight, and percent fat were obtained using an analytical balance (Mettler, Toledo, Osaka, Japan) and dual energy X-ray absorptiometry for rodents (Muromachi, Tokyo, Japan). Epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) were dissected, immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Each mouse was chronically implanted with a silastic catheter (No. 00; Shinnetsu, Tokyo, Japan) for serum sample collection. A catheter was inserted through the right jugular vein with the inner end placed just outside the right atrium. The sampling tube was attached to a 30-gauge Multi Sampling Needle to prevent air from being sucked into the system. Details on the sampling procedures were described elsewhere (30). Samples taken through the catheter were separated into serum and immediately frozen at -20°C until their use for assay. Serum glucose, insulin, and triglyceride were taken after overnight fasting to assay with commercially available kits (Eiken Chemical, Tokyo, Japan).

Load of mice with high- or low-fat diet. Matched for body weight at 8 weeks of age, H1KO and WT mice were divided into high-fat diet (HFD) and low-fat diet (LFD) groups (n = 6 for each subgroup). The HFD consisted of 45% fat, 35% carbohydrate, and 20% protein, with an energy density of 4.73 kcal/g. The LFD consisted of 10% fat, 70% carbohydrate, and 20% protein, with an energy density of 3.85 kcal/g. Body weight in each subgroup was measured weekly from 8 to 16 weeks of age. Total fat weight, percent fat, and *ob* gene expression in WAT were measured at 16 weeks of age.

Chronic implantation with a cannula into the lateral ventricle. Male adult mice at 12–14 weeks of age were anaesthetized with intraperitoneal injection of nembutal (1 mg/kg). Mice were placed in a stereotactic device to implant a 29-gauge stainless steel cannula chronically into the left lateral cerebroventricle (0.5 mm posterior, 1.0 mm lateral, and 2.0 mm ventral to the bregma). After surgery, a 30-gauge wire plug was inserted into each cannula to prevent blood coagulation. All the mice were allowed 1 week of postoperative recovery before they were handled daily to equilibrate their arousal levels. After cessation of all experiments, cannula placement was verified in each mouse infused with the dye Indiana green.

Procedures of leptin treatment. Murine recombinant leptin (Amgen, Thousand Oaks, CA) was dissolved in phosphate-buffered saline (PBS). To acquire a dose-response relationship, leptin was infused into the left lateral cerebroventricle at doses of 0.1, 0.25, and 0.5 μg/mouse daily for 3 successive days. The procedures of PBS infusion in the control group were the same as those in the leptin group, where applicable. The intracerebroventricular infusion volume of leptin and PBS was 0.1 μl. Matched according to basal body weight at 12–14 weeks of age, H1KO and WT mice were divided into the leptin and control groups (n = 6 for each). On the day before and for 3 days after treatment, food intake was measured daily in each subgroup (n = 6 for each subgroup). To prevent a difference in food consumption between the leptin and control groups, the control mice in each leptin infusion study were pair-fed daily with the appropriate leptin-treated mice. After the feeding evaluation, adipose tissues were surgically removed according to the procedures mentioned above and analyzed for fat accumulation and *UCP* expression.

Preparation of cDNA probe. Polymerase chain reaction (PCR) primers of 5′-CATCTTCTGGGA-GGTAGC-3′ and 5′-AAGACAGGGCAGGAATGG-3′ were designed to the coding region of the *UCP2* gene. Primers of 5′-GTTACCTTT CCACTGGACAC-3′ and 5′-CCGTTTCAGCTGCTCATAGG-3′ were designed to the *UCP3* gene. Reverse transcription of 10 μg total RNA from C57BL/6J mice was performed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD. PCR was carried out with Taq DNA polymerase (Amersham International, Buckinghamshire, England) and 20-pmol primers. The reaction profile was as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, for 30 cycles. The PCR fragment was subcloned into pCRTM2.1 vector (TA cloning kit; Invitrogen, San Diego, CA), and the nucleotide sequence of amplified cDNA was confirmed by sequencing. The nucleotide sequences were determined by the dideoxynucleotide chain termination method using synthetic oligonucleotide primers, which were complementary to the vector sequence, and ABI373A, automated DNA Sequencing System (Perkin-Elmer, Norwalk, CT). All DNA sequences were confirmed by reading both DNA strands. The *UCP-1*, *ob*, neuropeptide Y (NPY), and proopiomelanocortin (POMC) (GenBank accession no. U63419, U18812, M15880, and AH005319) probes were generated in an analogous fashion.

RNA extraction and Northern blot analysis. Total cellular RNA was prepared from various mouse tissues with the use of Isogen (Nippon gene, Toyama, Japan) according to the manufacturer's protocol. Total RNA (20 μg) was electrophoresed on 1.2% formaldehyde-agarose gel, and the separated RNA was transferred onto a Biodyne B membrane (Pall Canada, Toronto, ON, Canada) in 20× sodium chloride–sodium citrate by capillary blotting and immobilized by exposure to ultraviolet light (0.80 J). Prehybridization and hybridization were carried out according to the manufacturer's protocol. Membranes were washed under high-stringency conditions. After washing the membranes, the hybridization signals were analyzed with the BIO-image analyzer BAS 2000 (Fuji Film Institution, Tokyo, Japan). The membranes were stripped by exposure to boiling 0.1% SDS and rehybridized with a ribosomal RNA that was used to quantify the amounts of RNA species on the blots.

Statistical analysis. All the data were expressed as the mean ± SE. The statistical analysis of difference was assessed by Sheffe's or repeated two-way analysis of variance (Figs. 1–4), and the unpaired t test for multiple comparisons was used where appropriate (Table 1, Fig. 1). To evaluate the dose-response curve as to the effects of intracerebroventricular leptin infusion on food intake, the Spearman's correlation coefficient by rank was carried out.

RESULTS

Effects of H_1-R deficiency on bodily growth, body fat accumulation, and serum glucose, insulin, and triglyceride. As shown in Fig. 1*A*, no difference was found in growth rate and body weight between H1KO and WT mice from the weaning period of 1 week of age up to 30 weeks of age. Food intake (Fig. 1*B*), body weight, fat deposition, and fasting serum concentration of glucose, insulin, and triglyceride measured at 12 and 30 weeks of age did not differ between H1KO and WT mice (Table 1).

Effects of H_1-R deficiency on brain neuropeptides, UCP, and *ob* genes. Figure 1*C* and *D* show changes in gene expression involved in regulation of energy intake and expenditure in H1KO and WT mice at 12 (Fig. 1*C*) and 30 (Fig. 1*D*) weeks of age. Compared with the WT controls, no significant change was found in H1KO mice at either parameter, including hypothalamic mRNA of NPY or POMC and expression of BAT *UCP-1*, WAT *UCP-2*, WAT *UCP-3*, or WAT *ob* gene.

Effect of high-fat loading on H1KO mice. Loading of both male H1KO and WT mice with an HFD for 8 weeks starting at 8 weeks of age increased body weight more than that of mice with LFD ($P < 0.01$ for each), whereas no significant difference was found in body weight between H1KO and WT mice loaded with the same diet (either HFD or LFD) (Fig. 2*A*). Mean daily food intake in H1KO mice did not differ from that in WT mice throughout loading of these diets (data not shown). The notable result was that H1KO mice loaded with HFD increased total fat weight and percent fat more than WT mice with HFD ($P < 0.01$ for each), although there was no difference between the two types of mice loaded with LFD in either parameter (Fig. 2*B* and *C*). In agreement with these results, expression of *ob* gene in epididymal WAT was upregulated in H1KO mice loaded with HFD compared with that in the corresponding WT controls ($P < 0.01$) (Fig. 2*D* and *E*). There was no difference in serum glucose between H1KO and WT mice loaded with HFD, but serum insulin was increased in H1KO mice loaded with HFD

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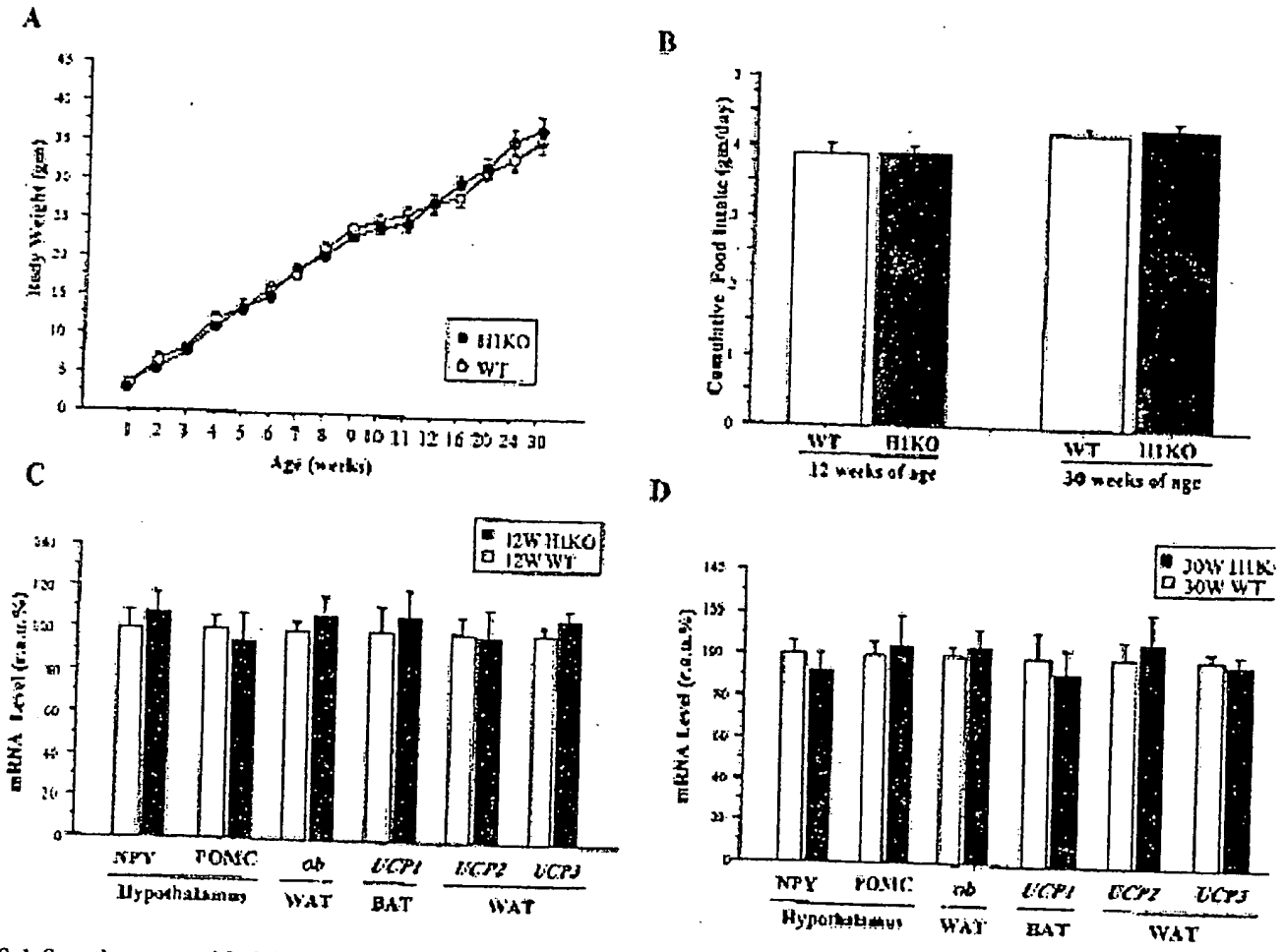

FIG. 1. Growth curves and 24-h food intake of H1KO and WT control mice (*A*). Note that no difference was found between H1KO and WT in the growth curve measured from weaning at 1 week of age up to 30 weeks of age or in daily food intake at 12 and 30 weeks of age (*B*). ues and vertical bars are means ± SE (n = 6 for each). *C* and *D*: Gene expression of NPY and POMC mRNAs in the hypothalamus, *UCP-1* n in BAT, *UCP-2* and *UCP-3* mRNAs in epididymal WAT, and *ob* mRNA in WAT in H1KO and WT mice at 12 (*C*) and 30 (*D*) weeks of age. N ern blot analysis was performed in total RNA (20 µg/lane) of the samples. No difference was found between the groups in either paran Values and vertical bars are means ± SE (n = 6 for each). r.a.u.%, Percent of relative arbitrary unit.

(202.1 ± 10.4 µU/ml) compared with corresponding WT controls (151.2 ± 8.3 µU/ml) ($P < 0.05$).

Effects of leptin infusion on food intake. The effect of central administration of leptin on 24-h food intake was investigated in H1KO and WT mice at 12–14 weeks of age. Intracerebroventricular infusion of 0.1–0.5 µg leptin/mouse daily produced dose-dependent feeding suppression (0.1, 0.25, and 0.5 µg leptin produced –9%, –16% and –34% food intake, res tively, compared with PBS controls; $r = 0.79$, $P < 0.01$). L infusion into the left lateral cerebroventricle at the hig dose of 0.5 µg/mouse daily for 3 days decreased food inta both H1KO and WT mice ($P < 0.01$ for each) (Fig. 3). Le induced feeding suppression was attenuated in H1KO compared with corresponding WT controls ($P < 0.05$) (Fi

TABLE 1
Body composition and serum concentration of mice at 12 and 30 weeks

Parameters	12 Weeks		30 Weeks	
	+/+	–/–	+/+	–/–
Body weight (g)	30.4 ± 1.1	30.3 ± 1.3	35.4 ± 1.2	36.1 ±
Body fat (g)	2.68 ± 0.28	2.85 ± 0.22	3.41 ± 0.30	3.75 ±
Body fat (%)	8.81 ± 0.92	9.40 ± 0.85	9.63 ± 0.84	10.39 ±
Glucose (mg/dl)	174.4 ± 15.3	167.5 ± 12.5	192.4 ± 15.1	190.0 ±
Insulin (µU/ml)	56.6 ± 7.6	59.3 ± 11.0	60.1 ± 8.7	69.8 ±
Triglyceride (mg/dl)	97.5 ± 9.4	96.8 ± 7.9	128.7 ± 11.4	138.9 ±

Data are means ± SE.

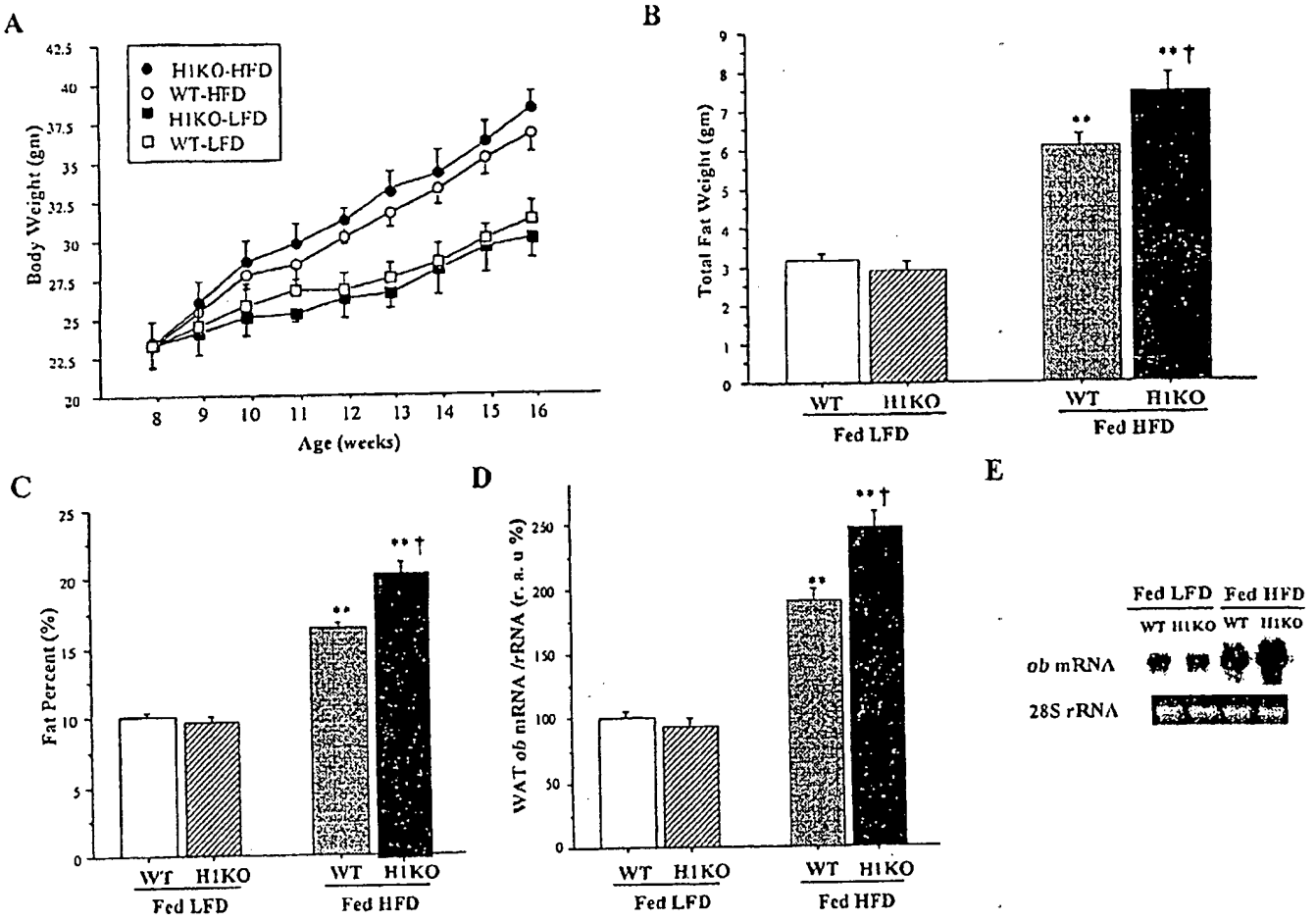

FIG. 2. Effects of HFD and LFD on body weight growth (*A*), fat weight (*B*), fat percent (*C*), and epididymal WAT *ob* gene expression (*D*, *E*) in H1KO and WT mice. Total fat weight, fat percent, and *ob* gene expression in H1KO mice fed with HFD were increased more than those in the corresponding WT mice. Values and vertical bars are means ± SE (n = 6 for each). r.a.u.%, Percent of relative arbitrary unit. $^{**}P < 0.01$ vs. corresponding LFD controls; $\dagger P < 0.05$ vs. corresponding WT controls.

Effect of intracerebroventricular leptin infusion on adiposity and *UCP* expression in BAT and WAT. Effects of chronic intracerebroventricular leptin infusion on adiposity and *UCP* expression in fat tissues were examined in pair-fed H1KO and WT mice at 12–14 weeks of age. Infusion of murine leptin into the left lateral cerebroventricle at a dose of 0.5 μg/mouse daily for 3 successive days lowered percent body fat in the pair-fed WT group compared with that in the corresponding controls with PBS ($P < 0.05$) (Fig. 4*A*). Leptin caused a more remarkable decrease of visceral fat weight than of subcutaneous fat (data not shown). The suppressive effect of leptin was attenuated in H1KO mice compared with that in the corresponding pair-fed WT controls ($P < 0.05$) (Fig. 4*A*). Gene expression of both *UCP-1* and *UCP-3* in BAT and *UCP-3* in WAT was upregulated more in both pair-fed H1KO ($P < 0.05$ for each) and WT mice ($P < 0.01$ for each) after intracerebroventricular leptin infusion than those in the corresponding PBS controls (Fig. 4*B* and *G*). Note that the accelerated effects of leptin on adiposity and *UCP* expression were attenuated more predominantly in H1KO mice than in the pair-fed corresponding WT controls ($P < 0.05$ for each) (Fig. 4*B* and *G*).

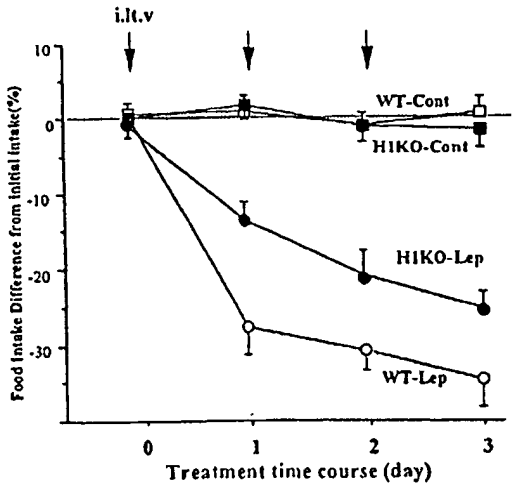

FIG. 3. Effect of leptin (Lep) infusion on food intake in H1KO and WT mice at 12–14 weeks of age. Suppressive effect of Lep on feeding was attenuated in H1KO mice with Lep. Values are the percent difference from corresponding initial baseline value. Values and vertical bars are means ± SE (n = 6 for each). The arrows indicate infusion of Lep or PBS (Cont) into the lateral cerebroventricle (i.lt.v.). See the text for details about significant differences between the subgroups.

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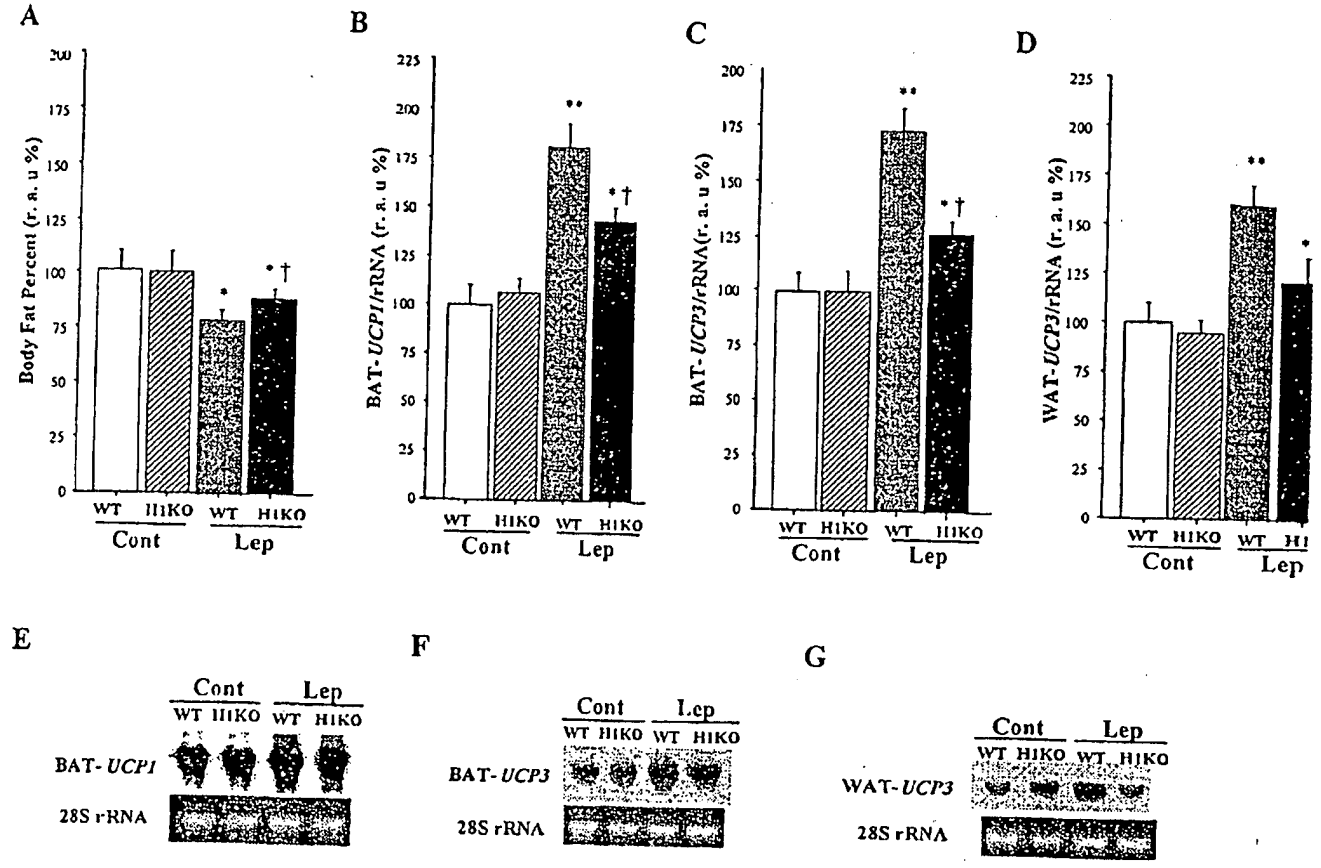

FIG. 4. Effect of leptin (Lep) infusion on fat percent (*A*) and *UCP* gene expression in H1KO and WT mice at 12–14 weeks of age. Each p eter was attenuated or enhanced in H1KO mice after Lep treatment compared with PBS controls (Cont). Values are the percent differ from the corresponding initial baseline value. Values and vertical bars are means ± SE (n = 6 for each). r.a.u.%, Percent of relative arb unit. *P < 0.05, **P < 0.01 vs. corresponding untreated controls; †P < 0.05 vs. corresponding WT controls. Gene expression of BAT *UCP-*. BAT *UCP-3* (*F*), and WAT *UCP-3* (*G*) in H1KO and WT mice after Lep or PBS (Cont) treatment.

DISCUSSION

The effects of histamine neurons on brain functions are mediated by histamine H_1-, H_2-, and H_3-R, which belong to a family of guanine nucleotide-binding regulatory G protein–coupled receptors (2). These histamine receptors are involved in a variety of physiological functions: mediation of immune response (31), circadian rhythm (29), and food intake through H_1-R (3,4); endocrine system and gastric secretion through H_2-R (32); and auto-inhibition for negative feedback regulation through H_3-R that affects histamine contents and turnover (33). The present study focused on the role of H_1-R in the leptin signaling pathway, which regulates feeding behavior and *UCP* expression.

Deficit in H_1-R per se has been found to produce no manifest change in gene expression of peripheral *UCP*s or hypothalamic neuropeptides such as NPY and POMC. Consistently, food consumption, growth rate, and body weight did not change in H1KO mice. These results are compatible with our previous finding that histamine depletion by chronic treatment with α-fluoromethylhistidine did not affect daily food intake or body weight growth, although acute depletion of neuronal histamine induced a transient increase in feeding (34). Together with the resulting and foregoing findings, it would be reasonable to argue that the effectiveness of histamine depletion or defect in H_1-R may not be detectable under normal conditions, because histamine neurons i hypothalamus appear to play homeostatic rather than d roles in energy balance (1). In this context, histamine neu in parallel with other regulatory systems of energy me lism may act indirectly to influence major physiologic d minants of energy balance. The assumption is supporte the present study on feeding mice an HFD. More increase in body fat deposition was induced in H1KO after access to HFD. Here, an essential question can be r as to why body weight did not differ between the WT H1KO groups, although the adipose parameters of tot weight, percent fat, and *ob* gene expression did c between those groups. There is no definite answer t query to date. One possible and reasonable explanation is the increase in body fat may be too small to affect weight. Another possibility is that the difference in r body weight gain between those groups is relatively s compared with the deviation of body weight in both gr

The resulting information indicates that a genetic defe histamine signaling through H_1-R is capable of enhancin development of adiposity leading to obesity. In normal ditions, histamine neurons function through H_1-R to pre the living body from excessive fat accumulation induce high-energy intake. There are four possible explanation the action of neuronal histamine against obesity dev

ment. First, neuronal histamine has been demonstrated to suppress food intake through H_1-R in the VMH and the PVN, both of which are known as satiety centers (3,4). However, the mechanism is not likely to have played a main role in the present study, because no significant difference was found in daily consumption of HFD between H1KO and WT mice. Second, histaminergic activation may lead to increased sympathetic outflow and lipolysis. Indeed, previous reports demonstrated increases in lipolytic responses together with elevation of serum free fatty acids through the sympathetic pathway (8,9). Third, the present study revealed that H1KO mice increased insulin levels in response to an HFD. The result indicates that besides sympathetic outflow to fat pads, HFD-induced insulin secretion in H1KO mice may promote lipogenesis in adipocytes because insulin increases leptin synthesis (35), as the present result showed WAT *ob* mRNA was upregulated. Finally, it seems quite reasonable that histamine neurons may be involved in central regulation of *UCP* expression as indicators of energy expenditure. *UCP-1* and *UCP-3* in BAT and *UCP-3* in WAT are upregulated by a β_3-agonist, indicating effects of sympathetic activity on their gene expression (36). Administration of leptin has been shown to affect BAT and WAT *UCP* mRNAs through the activation of sympathetic nerve (28,37). BAT UCP-1 is shown modulated not only by sympathetic nerve activity (20,38) but by upper brain function (28,39). Under this scenario, histamine neurons may guard against fat accumulation, depending on histamine-induced upregulation of *UCP*s.

The question arises as to what kind of information activates histamine neurons in response to HFD loading. Leptin is a most probable candidate for the activation. HFD-promoted fat accumulation leads to elevation of leptin secretion from expanded adipose tissues (40,41). Thereafter, leptin activates histamine release in the hypothalamus and limits food intake (11). Ultimately, the activation of histamine neurons drives a negative feedback loop that downregulates adiposity and feeding by returning them to those initial levels. Blockade of the histamine signaling pathway at an H_1-R level may limit leptin's actions on food intake and regulation of *UCP* expression. It would be very much in line with our present results indicating that the signaling pathways of leptin interact with those of histamine neurons in the regulation of feeding, adiposity, and *UCP* expression. Notably, the suppressive effects of leptin on feeding behavior and fat accumulation and the accelerating effects of leptin on *UCP* expression were partially attenuated in H1KO mice. Taken together with the forgoing explanation of leptin-induced *UCP* upregulation (28,37) and the interaction of leptin signaling messages with those of histamine neurons (11), this attenuation may result from reduced sympathetic nerve activity. The present study demonstrates that histamine neurons play an important role in the leptin signaling pathway through H_1-R. Coordinate interactions of histamine neuron systems with leptin signaling thus regulate suppression of feeding behavior, acceleration of lipolysis, and upregulation of the *UCP* family.

Evidence has rapidly emerged to indicate that development of obesity results from interaction between genetic and environmental factors. Developmental patterns of H1KO mice when fed an HFD resemble middle-aged human obesity. More recently, brain serotonin systems were reported to have a causal relation with fat deposition in middle-aged and diet-induced obesity (42). In this context, a genetic defect in histamine signaling transduction and/or the intake of a high-fat, high-energy diet tend to develop obesity. It is not surprising for the foregoing reasons that the inhibition of brain histamine neurons will predispose animal models and humans to obesity.

In conclusion, H1KO mice are not only useful as a new leptin-resistant model but also provide a novel insight that H_1-R contributes as a key receptor in the regulation of energy homeostasis when mice are fed an HFD and acts as a downstream signal of leptin's actions in the brain.

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Behavioral Characterization of Mice Lacking Histamine H_3 Receptors

HIROSHI TOYOTA, CHRISTINE DUGOVIC, MURIEL KOEHL, AARON D. LAPOSKY, CHINA WEBER, KAREN NGO, YING WU, DOO HYUN LEE, KAZUHIKO YANAI, EIKO SAKURAI, TAKEHIKO WATANABE, CHANGLU LIU, JINGCAI CHEN, ANN J. BARBIER, FRED W. TUREK, WAI-PING FUNG-LEUNG, and TIMOTHY W. LOVENBERG

Johnson & Johnson Pharmaceutical Research and Development, San Diego, California (H.T., K.N., Y.W., D.H.L., C.L., J.C., A.J.B.,W.-P.F.-L., T.W.L.); Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois (C.D., M.K., A.D.L., C.W., F.W.T.); and Department of Pharmacology, Tohoku Graduate School of Medicine, Tohoku, Japan (K.Y., E.S., T.W.)

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ABSTRACT

Brain histamine H_3 receptors are predominantly presynaptic and serve an important autoregulatory function for the release of histamine and other neurotransmitters. They have been implicated in a variety of brain functions, including arousal, locomotor activity, thermoregulation, food intake, and memory. The recent cloning of the H_3 receptor in our laboratory has made it possible to create a transgenic line of mice devoid of H_3 receptors. This paper provides the first description of the H_3 receptor-deficient mouse ($H_3^{-/-}$), including molecular and pharmacologic verification of the receptor deletion as well as phenotypic screens. The $H_3^{-/-}$ mice showed a decrease in overall locomotion, wheel-running behavior, and body temperature during the dark phase but maintained normal circadian rhythmicity. $H_3^{-/-}$ mice were insensitive to the wake-promoting effects of the H_3 receptor antagonist thioperamide. We also observed a slightly decreased stereotypic response to the dopamine releaser, methamphetamine, and an insensitivity to the amnesic effects of the cholinergic receptor antagonist, scopolamine. These data indicate that the H_3 receptor-deficient mouse represents a valuable model for studying histaminergic regulation of a variety of behaviors and neurotransmitter systems, including dopamine and acetylcholine.

The neurotransmitter histamine, which originates from tuberomamillary nuclei in the posterior hypothalamus, projects diffusely throughout the central nervous system (CNS) and has been implicated in the regulation of many functions, including sleep/wake, food and water intake, thermoregulation, memory, and other homeostatic processes (Wada et al., 1991; Brown et al., 2001). Four subtypes (H_1, H_2, H_3, and H_4) of histamine receptors are currently recognized (Hill et al., 1997; Hough, 2001). The H_3 subtype is predominantly located presynaptically and serves as an autoreceptor to regulate the synthesis and release of histamine (Hill et al., 1997). The H_3 subtype also has heteroreceptor functions and influences CNS dopamine, γ-aminobutyric acid, noradrenaline, acetylcholine, and serotonin levels (Arrang et al., 1983, 1987b; Schlicker et al., 1988; Clapham and Kilpatrick, 1992; Hill et al., 1997). Behavioral correlates of H_3 receptor function have primarily been studied in the context of pharmacologically blocking the receptor using the specific H_3 receptor antagonist, thioperamide. For instance, thioperamide has been used to increase the amount of wakefulness (Monti et al., 1991), to prevent scopolamine-induced amnesia (Giovannini et al., 1999), and to decrease food intake (Itoh et al., 1999; Attoub et al., 2001) in rats. The recent cloning of the H_3 receptor in our laboratory (Lovenberg et al., 1999) has made it possible to create a transgenic line of mice devoid of H_3 receptors and to explore at a molecular level the importance of this receptor in a variety of behaviors. This paper provides the first description of 1) generating the H_3 receptor knockout mice, 2) verifying the deletion with radioligand binding and a pharmacologic challenge, and 3) testing the neurochemical and behavioral consequences of deletion of the H_3 receptor.

Materials and Methods

Generation of Histamine $H_3^{-/-}$ Receptor Animals

Mouse H_3R gene clones were isolated from a 129/Ola mouse genomic library, and phage clones covering 13 kilobases of the mouse H_3R gene were isolated. The *Xho*I DNA fragments containing the second exon of the mouse H_3R gene were used to prepare the knockout construct. A cassette containing a neomycin resistance gene was used to replace a 0.7-kilobase region covering part of the first intron

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H.T. and C.D. contributed equally to this study.

ABBREVIATIONS: CNS, central nervous system; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; HPLC, high performance liquid chromatography; LD, light/dark; EEG, electroencephalogram; EMG, electromyogram; NREM, non-rapid eye movement; REM, rapid eye movement; ANOVA, analysis of variance; DD, constant darkness; MAP, methamphetamine.

and the 5' end of the second exon of the gene. A HSV-thymidine kinase cassette was placed at the 3' end of the construct. The mouse H_3R gene, the neomycin resistance gene, and the HSV-thymidine kinase gene in the construct are in the same orientation of transcription. The DNA construct was introduced into embryonic day 14 embryonic stem cells by electroporation. Cells were cultured in the presence of 400 μg/ml geneticin (G418) and 0.2 μM ganciclovir. Embryonic stem cells with the disrupted gene were detected by polymerase chain reaction (PCR) and then confirmed by Southern hybridization using a DNA probe flanking the 3' end of the construct. Chimeric mice were generated from embryos injected with embryonic stem cells. Germline mice were obtained from breeding of chimeric male mice with C57BL/6J females. Germline mice heterozygous for the disrupted H_3R gene were identified by PCR. H_3R-deficient mice carrying only the disrupted H_3R gene were obtained from cross-breeding of heterozygous mice. Wild-type and homozygous animals were produced from the breeding of the germline heterozygotes. These wild-type and homozygous mice were further bred to produce the animals used for this study.

Northern Blot and RT-PCR

Northern Blot Hybridization. Twenty micrograms of total RNA from $H_3^{+/+}$ and $H_3^{-/-}$ mouse brains were run on a formaldehyde gel and transferred onto a Nytran nylon membrane (Schleicher & Schuell, Keene, NH). The membrane was prehybridized with hybridization buffer: 50% formamide, 5× sodium chloride/sodium phosphate/EDTA, 5× Denhardt's solution, 0.1% SDS, and 200 μg/ml of single-stranded DNA. The membrane was then hybridized overnight at 42°C with a ^{32}P-labeled probe against mouse H_3 exon 3 DNA. As control, a mouse beta actin DNA probe was used in a parallel experiment.

Reverse-Transcriptase PCR. Total RNA was isolated from $H_3^{-/-}$ and $H_3^{+/+}$ mouse brains using the TRIzol RNA purification kit (Invitrogen, San Diego, CA). cDNAs were synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) and random primers as described by the manufacturer. PCR was used to detect the H_3 mRNA expression with brain cDNA from either $H_3^{-/-}$ or $H_3^{+/+}$ mouse as templates, and with P1: 5'-CTCTGCAAGCTGTGGCTGGTGGTAGACTACCTACTGTGTG-3' and P2: 5'-CTTCTTGTCCCGCGACAGCCGAAAGCGCTGGGTGATGCTT-3' as primers. The PCRs were performed under conditions of 94°C, 40 s; 65°C, 40 s; 72°C, 2 min for 40 cycles. As control, mouse glycerol-3-phosphate dehydrogenase primers (BD Biosciences Clontech, Palo Alto, CA) were used to amplify glycerol-3-phosphate dehydrogenase cDNA in a parallel PCR reaction. The PCR products were run in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Measurement of Histamine H_3 and H_1 Receptor Binding

Preparation of Membrane Fraction. Mice were euthanized by gas (CO_2) and decapitated immediately. Brains were removed and stored at −80°C until used further. Forebrain tissue was homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in ice-cold 50 mM Na/K phosphate buffer, pH 7.5. The homogenates were centrifuged twice at 50,000*g* for 20 min at 4°C. The resulting pellets were resuspended in binding buffer and kept at 4°C until needed.

The Histamine H_3 Binding Assay. The histamine H_3 receptor binding was assayed by a modification of the method of Arrang et al. (1987a). Briefly, to examine inhibition curves, 0.4-ml aliquots of the membrane suspensions (10 mg of tissue) were incubated for 60 min at 25°C with 1.5 nM (*R*)-[α-^{3}H] methylhistamine. Specific binding was defined as that inhibited by 10 μM thioperamide. The reaction was terminated by addition of 5 ml of the ice-cold buffer and rapid filtration on a glass fiber filter (GF/B) precoated with 0.3% polyethyleneimine. The filters were washed three times with 5-ml volumes of the ice-cold buffer, and the radioactivity trapped on the filters was counted in 10 ml of ScintiVerse (Fisher Scientific, Atlanta, GA).

The histamine H_1 binding was assayed by a modification of the method of Tran et al. (1978). In brief, 25 μg of brain membrane was used for the ligand-binding assay with the H_1 receptor antagonist, [^{3}H]pyrilamine and with 1 μM quinine to prevent binding to the cytochrome P450-like protein (Liu et al., 1994) (25°C). Concentrations of [^{3}H]pyrilamine were used for Scatchard analysis. Nonspecific binding was determined in the presence of 50 μM triprolidine, an H_1 receptor antagonist. The samples were counted as described above.

Measurement of Neurotransmitter Contents

After decapitation, brains were removed rapidly and divided into the cortex and cerebellum. The brain tissues were stored at −80°C until assayed. Brain tissue was homogenized in 3% perchloric acid containing 5 mM disodium EDTA and 5-hydroxy-*N*$^{\omega}$-methyltryptamine by a Polytron homogenizer (Kinematica) at a maximum setting for 10 s in an ice bath, and the homogenate was centrifuged at 10,000*g* for 10 min at 4°C to obtain a clear supernatant. The histamine content was measured fluorometrically with *O*-pthalaldehyde (Shore et al., 1959) after separation on an HPLC system as described by Yamatodani et al. (1985). Briefly, histamine was separated on a cation exchanger, TSK gel SP2SW9 (Tosoh, Tokyo, Japan; particle size 5 μm), eluted with 0.25 M KH_2PO_4 at a flow rate of 0.6 ml/min using a constant flow pump (model CCPM; Tosoh). The histamine eluate was derivatized using an on-line automated Shore's *O*-pthalaldehyde method (Shore et al., 1959), and the fluorescence intensity was measured at 450 nm with excitation at 360 nm in a spectrofluorometer equipped with a flow cell (model C-R3A; Shimadzu, Kyoto, Japan) and a chromatographic data processor.

Monoamine content (dopamine, homovanillic acid, 3,4-dihydroxyphenylacetic acid, noradrenaline, serotonin, and 5- hydroxyindolacetic acid) in brain samples was measured with an HPLC system with an electrochemical detector (Yanai et al., 1998). They were separated using an HPLC system at 30°C on a reverse-phase analytical column (DS-80TM, 4.6 mm i.d. × 15 cm) and detected by an electrochemical detector (model ECD-100; Eikom Co, Kyoto, Japan). The column was eluted with 0.1 M sodium acetate-citric acid buffer, pH 3.5, containing 15% methanol, 200 mg/l sodium L-octanesulfate, and 5 mg/l disodium EDTA. All measurements were blinded with respect to brain structures and subject group.

Recording of Locomotor Activity, Body Temperature, and EEG/Waking Response to Thioperamide

Electrode and Transducer Implantation. At 3 months of age, 10 $H_3^{+/+}$ and 10 $H_3^{-/-}$ male (24–34 g) mice were implanted with chronic electrodes for polygraphic recording of frontoparietal electroencephalogram (EEG) and nuchal electromyogram (EMG) under deep anesthesia (i.p. injection of ketamine/xylazine). The implant consisted of two stainless steel screws (1-mm diameter) inserted through the skull, serving as EEG electrodes, and two insulated wires inserted into the nuchal muscles, serving as EMG electrodes. All electrodes were attached to a microconnector and fixed to the skull with dental acrylic cement. In addition, transducers (PDT-4000 E-Mitter, Mini-Mitter; Minimitter Co., Bend, OR) were inserted through a small incision off midline in the peritoneal cavity for biotelemetric recording of locomotor activity and body temperature. Aftersurgery, mice were individually housed and given 2 weeks to recover from the procedure.

For behavioral tests of locomotor, body temperature, and EEG/EMG activity, it is necessary to individually house animals, which may be considered a stressor. To minimize stress as a confounding variable, animals were carefully adapted to the housing and recording environments before data collection, and each genotype was treated similarly under all experimental conditions.

Baseline Recordings of Locomotor Activity and Body Temperature. After 2 weeks of recovery from surgery, the mice were transferred to a recording chamber for 2 to 3 days of adaptation

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followed by 48-h recording of locomotor activity and body temperature. The recording environment was temperature (23–24°C)- and light [12-h/12-h light/dark (LD) cycle]-controlled, with food and water available ad libitum. The biotelemetry transducers were precalibrated to produce radiofrequency signals indicating locomotor counts (number of movements per 10-s time period) and body temperature (accurate to 0.1°C). The transducers were powered by an induction coil, and output signals were detected by a radiofrequency receiver placed under each mouse cage. Data were collected using a software package developed in our laboratory (Multisleep 5.01; Actimetrics, Evanston, IL) for the analysis of EEG/EMG and circadian rhythm measurements.

Thioperamide Challenge. After 2 weeks of recovery from surgery, $H_3^{+/+}$ (n = 6) and $H_3^{-/-}$ (n = 7) mice were connected to a cable/rotating swivel system for EEG/EMG recording and allowed 1 week of adaptation to a sleep recording chamber. Each mouse was then injected with saline subcutaneously (4 ml/kg body weight) at the onset of the light phase followed by 6 h of EEG/EMG recording. The next day, again at the onset of the light phase, each mouse was given 10 mg/kg thioperamide (dissolved in saline) subcutaneously, and EEG/EMG activity was collected for 6 h. EEG/EMG signals were fed into amplifiers (Grass model 12; Astro-Med Grass Instrument Division, West Warwick, RI). The signals were then digitized and stored on an on-line computer data acquisition program (Multisleep 5.01; Actimetrics Inc., Evanston, IL).

Data Analysis. Polygraphic recordings were visually scored by 10-s epochs as either wake (W), non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep, according to standard criteria. Briefly, the different vigilance states were characterized as follows: W, low-voltage mixed frequency EEG activity and high EMG activity; NREM, continuous high amplitude low frequency activity in EEG and low EMG activity; and REM, low-voltage fast frequency cortical waves with a regular theta rhythm and absence of muscular tone. Sleep-wake parameters were analyzed over 2-h time intervals. The duration (minutes) of time spent in the different states of vigilance was expressed as a ratio of the total recording session duration (percentage of total recording time). Locomotor activity and body temperature were analyzed over 1-h time intervals, as well as over the 12-h light and dark phases and the total 24-h periods.

Data were analyzed using two-way repeated measures ANOVAs for between (genotype)- and within (time of day)-group factors. Significant interactions were followed up using Newman-Keuls post hoc analyses.

Acute Response of Locomotion and Stereotyped Behavior to Methamphetamine

Locomotion. $H_3^{+/+}$ and $H_3^{-/-}$ mice were injected with biological saline [0.9% (w/v) NaCl] or methamphetamine (1mg/kg i.p). Locomotor activity was measured with a photo-beam system (Hamilton-Kinder MotorMonitor System SmartFrame Cage Rack system with two-dimensional 4 × 8 beam; Hamilton-Kinder LLC, Julian, CA). Values for locomotor activity were calculated every 20 min, from 60 min before the injection of vehicle or methamphetamine until 180 min after the injection, and recorded with a personal computer. We used rat cages (9 × 16.93 × 7.87 inches) with a flat top grid that prevented the mice from climbing to the top lid. We used a floor grid with little bedding, which prevented the mice from making nests that could cut the photo-beam. The scores were analyzed by a two-way repeated ANOVA followed by a Student-Newman-Keuls test and a one-way ANOVA followed by Duncan's test.

Stereotyped Behavior. $H_3^{+/+}$ and $H_3^{-/-}$ mice were injected with biological saline [0.9% (w/v) NaCl] or methamphetamine (1 mg/kg i.p.). Stereotyped behavior was rated every 20 min, from 60 min before injection of the drug until 180 min after injection, according to the scale described by McLennan and Maier (1983). Scores were defined as follows: 0 = inactive; 1 = intermittent activity; 2 = continuous activity; 3 = intermittent stereotypy; 4 = continuous stereotypy over a wide area including stereotyped locomotor activity, sniffing, and rearing; 5 = continuous stereotypy over a restricted area (mainly sniffing and rearing); 6 = pronounced continuous stereotypy in a restricted area (mainly sniffing); 7 = intermittent licking or biting; and 8 = continuous licking or biting. The scores were analyzed by a two-way repeated ANOVA followed by a Student-Newman-Keuls test and a one-way ANOVA followed by Duncan's test.

Open Field Habituation

Open field habituation was assessed by a modification of the method of Molinengo et al. (1999). In brief, animals were placed individually in an open field apparatus (50 × 50 cm), and their locomotion distance was measured for 5 min using a videotracking system. Animals were tested for three consecutive days in the same activity chambers. To evaluate the effect of repetition of the test, the ambulation ratios (percentages) of the second and third days against the first day in the open field were calculated. Scopolamine (0.75 mg/kg) or vehicle (saline) was given intraperitoneally 30 min before the first and second test.

Wheel Running Activity

$H_3^{+/+}$ (n = 8) and $H_3^{-/-}$ (n = 11) mice were individually housed in a 33- × 15- × 13-cm polycarbonate cage (Nalge Nunc International, Naperville, IL) equipped with a 12.5-cm-diameter stainless steel exercise wheel. Each wheel revolution triggered a microswitch (Cherry Electric, Pleasant Prairie, WI) mounted on the outside of the cage near the axle of the wheel. The trigger closed an electrical circuit, and these resistance changes indicated the number of wheel revolutions per minute. Data were collected by a DOS PC computer system (Chronobiology Kit; Stanford Software Systems, Stanford, CA). This software generates data output in numeric and graphic form.

Each mouse was recorded under a 12:12-h LD schedule followed by a session in constant darkness (DD). The data represent the number of wheel revolutions during the last 5 days of exposure to LD, before the animals were transferred to DD, and wheel revolutions over five consecutive circadian cycles after they had been exposed to DD for 10 days. Because both genotypes have similar circadian periods, the absolute interval of time for determining the number of wheel revolutions was the same in DD.

LD data were analyzed using two-way repeated measures ANOVA and Newman-Keuls post hoc tests to resolve interaction effects. Data from DD trials were analyzed using an independent-samples Student's t test for genotype comparisons.

Passive Avoidance Test

The apparatus consisted of two compartments, one (9.5 × 18.5 × 16 cm) being surrounded by a white wall and illuminated by a 60-W lamp, and the other (9.5 × 18.5 × 16 cm) being dark and surrounded by a black wall. The compartments were separated by a guillotine door (4.5 × 4.5 cm). All the mice were habituated to the dark chamber for 60 min before the test. On the first day of the passive avoidance test, $H_3^{+/+}$ and $H_3^{-/-}$ mice were divided into two groups. One group of $H_3^{+/+}$ (n = 14) and $H_3^{-/-}$ (n = 18) mice was injected with scopolamine (0.75 mg/kg i.p., 30 min before the session), whereas the other group of $H_3^{+/+}$ (n = 13) and $H_3^{-/-}$ (n = 14) mice was injected with biological saline, 0.9% (w/v) NaCl, (1 mg/kg i.p.). The mice were placed into the illuminated safe compartment for 30 s before being given free access to the dark box. The mice tended to escape into the dark compartment. When all four paws were on the grid floor of the dark compartment, a scrambled constant-current foot shock (1 mA, constant voltage 120 V, 50 Hz) was delivered to the grid for 1 s. Then the mice were returned to their home cages. Twenty-four hours later, the procedure, without the electric shock, was repeated. The time that elapsed before each mouse entered the dark compartment was measured. The latency value of 300 s was

assigned when animals did not enter the dark compartment within 300 s. The results were analyzed by Student's *t* test.

Results

Confirmation of Successful Knockout of the Histamine H_3 Receptor Gene. Mice lacking the H_3 receptor gene ($H_3^{-/-}$) were created via homologous recombination in embryonic stem cells (129SVJ), and germline chimeras were crossed onto a C57BL/6J background to generate heterozygotes ($H_3^{+/-}$). $H_3^{+/+}$ and $H_3^{-/-}$ mice were created by breeding of $H_3^{+/-}$ mice, and germline transmission was determined by polymerase chain reaction. F2 $H_3^{-/-}$ mice were born with an expected mendelian frequency, appeared phenotypically normal, were fertile, and appeared viable through adulthood. Growth curves for $H_3^{+/+}$ and $H_3^{-/-}$ were parallel with the $H_3^{-/-}$ animals displaying a slightly lower, but not statistically significantly different, average body weight (not shown). The total absence of H_3 receptors in the transgenic mice was verified by Northern blot, RT-PCR, and radioligand binding studies (Fig 1). Whereas H_3 receptors can be readily detected via radioligand binding in normal ($H_3^{+/+}$) mouse brain homogenates, $H_3^{-/-}$ mice demonstrated a complete loss of H_3 receptor binding sites as determined by (*R*)-[α-^{3}H]methylhistamine binding (Fig. 1B). Heterozygous mice ($H_3^{+/-}$) had the same (*R*)-[α-^{3}H] methylhistamine binding affinity (0.43 nM) as the $H_3^{+/+}$ mice (0.47 nM), but only about half the number of binding sites in whole brain homogenates (47 fmol/mg of protein versus 91 fmol/mg of protein, respectively).

$H_3^{-/-}$ Mice Have Normal Brain Levels of Dopamine, Norepinephrine and Serotonin, but Decreased Levels of Histamine. We compared the brain levels of several neurotransmitters in $H_3^{+/+}$ and $H_3^{-/-}$ mice. Neurotransmitter content of the cerebral cortex was measured and no significant differences were found for dopamine ($H_3^{+/+}$ = 1.79 ± 0.28 nmol/g versus $H_3^{-/-}$ = 1.59 ± 0.16 nmol/g), norepinephrine ($H_3^{+/+}$ = 0.37 ± 0.04 nmol/g versus $H_3^{-/-}$ = 0.37 ± 0.04 nmol/g), or serotonin ($H_3^{+/+}$ = 0.57 ± 0.13 nmol/g versus $H_3^{-/-}$ = 0.47 ± 0.11 nmol/g), or any of their metabolites (not shown). However, significant differences were observed for cortical histamine content ($H_3^{+/+}$ = 266.8 ± 45.1 pmol/g versus H3$^{-/-}$ = 156.1 ± 35.5 pmol/g, p <0.05).

$H_3^{-/-}$ Mice Have Decreased Spontaneous Locomotor Activity and Wheel Running Behavior. To assess the effects of the H_3 receptor on circadian rhythmicity and activity levels, both total locomotor activity and wheel running were evaluated. Significant interactions between genotype and time of day were detected for locomotor activity (day 1, $F = 2.33(1,23), p < 0.001$; day 2, $F = 2.14(1,23), p < 0.01$) and body temperature (day 1, $F = 1.95(1,23), p < 0.01$; day 2, $F = 2.11(1,23), p < 0.01$). The results of follow-up tests showed that the $H_3^{-/-}$ mice had markedly decreased locomotor activity during the dark phase of the circadian cycle (Fig 2a), and this was reflected by an impaired temperature elevation during the dark phase (Fig. 2B). Although there was no significant difference in the number of wheel running revolutions during the light phase, the significant dark phase effect ($F = 4.33(1,17), p < 0.05$) resulted in an overall 22% decrease in wheel running behavior over the 24-hr light/dark cycle in the $H_3^{-/-}$ mice (Fig. 2C). This overall decrease in running wheel behavior persisted in continuous darkness where on average there was about a 24% decrease in the number of wheel revolutions over the circadian cycle in the $H_3^{-/-}$ mice (t = 2.32, df = 14, $p < 0.05$) (Fig. 2C). It is interesting to note that whereas the amplitude of the changes in locomotor activity and body temperature was blunted, circadian rhythmicity was maintained. We found no difference in either the phase angle of entrainment of the activity rhythm to the light/dark cycle, nor were there any differences in the free running period of the activity rhythm (period = 23.6 ± 0.1 h in $H_3^{+/+}$ animals and 23.5 ± 0.1 h in $H_3^{-/-}$ mice).

The $H_3^{-/-}$ Mice Are Insensitive to the Wake-Promoting Effect of Thioperamide. To test the role of the H_3 receptor in mediating arousal states, we compared the wake-promoting effects of the H_3 receptor antagonist, thioperamide in $H_3^{+/+}$ and $H_3^{-/-}$ mice. A significant interaction between genotype and drug occurred for percentages of wakefulness ($F = 29.59(1,11), p < 0.001$) and NREM sleep ($F = 32.63(1,11), p < 0.001$). Follow-up testing (Fig 3) showed that thioperamide increased waking by 55% during the first 2 h after administration (at lights-on) in $H_3^{+/+}$ mice. This increase in waking was associated with a 61% decrease in NREM sleep during the 2-h postinjection period, whereas REM sleep was not affected (Fig. 3). Importantly, there were no effects of thioperamide on the sleep-wake status of the $H_3^{-/-}$ mice, providing behavioral confirmation that the receptor had been deleted, and verifying that this behavioral effect of thioperamide is indeed mediated through the H_3 receptor.

The $H_3^{-/-}$ Mice Show a Decreased Sensitivity to Methamphetamine. We examined the response of the $H_3^{-/-}$ mice to methamphetamine (1 mg/kg), which is known to increase locomotor activity and to induce stereotypic behavior, in part, by increasing dopamine release (Grilly and Loveland, 2001). After injections of methamphetamine, the decline of the stereotypy scores was faster in $H_3^{-/-}$ compared with $H_3^{+/+}$ mice [$F = 31.348(3,24), p < 0.001$]. One-way ANOVA followed by Duncan's test at every time point showed that methamphetamine-injected $H_3^{-/-}$ mice had significantly lower stereotypy scores than $H_3^{+/+}$ mice between 60 and 160 min (Fig. 4A). Likewise, there was an overall difference in the methamphetamine-induced locomotion between the $H_3^{+/+}$ and $H_3^{-/-}$ mice [$F = 4.051(3,24), p < 0.001$]. A one-way ANOVA followed by Duncan's test indicated that the ambulation of methamphetamine-injected $H_3^{-/-}$ mice was significantly lower than that of $H_3^{+/+}$ mice between 80 and 120 min after the injection. These data indicate that $H_3^{-/-}$ mice recover faster from methamphetamine-induced locomotion and stereotypy than $H_3^{+/+}$ mice.

The $H_3^{-/-}$ Mice Are Resistant to the Amnesic Effect of Scopolamine in the Passive Avoidance Test. The effect of deletion of the H_3 receptor on memory function was investigated using the step-through passive avoidance test. This test uses a light/dark preference and an acute aversive conditioning stimulus (mild foot shock), and has been used to demonstrate cognitive/memory enhancement of cholinesterase inhibitors. In a basic light/dark distribution test, there was no difference between $H_3^{+/+}$ and $H_3^{-/-}$ mice either in time spent in the light or dark compartments or in the number of transitions from light to dark compartment (data not shown). There was no difference between $H_3^{+/+}$ and $H_3^{-/-}$ mice in the basic passive avoidance test as both sets of mice

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were equally able to retain the recollection of the aversive stimulus (Fig. 4B). When the $H_3^{+/+}$ mice were pretreated with the amnesic agent scopolamine (a muscarinic receptor antagonist) before their first exposure to the chamber, they failed to recall the aversive stimulus upon reintroduction to the chamber on the next day (Fig. 4B, right). However, the $H_3^{-/-}$ mice were completely unresponsive to the amnesic effects of scopolamine and responded similarly to the un-

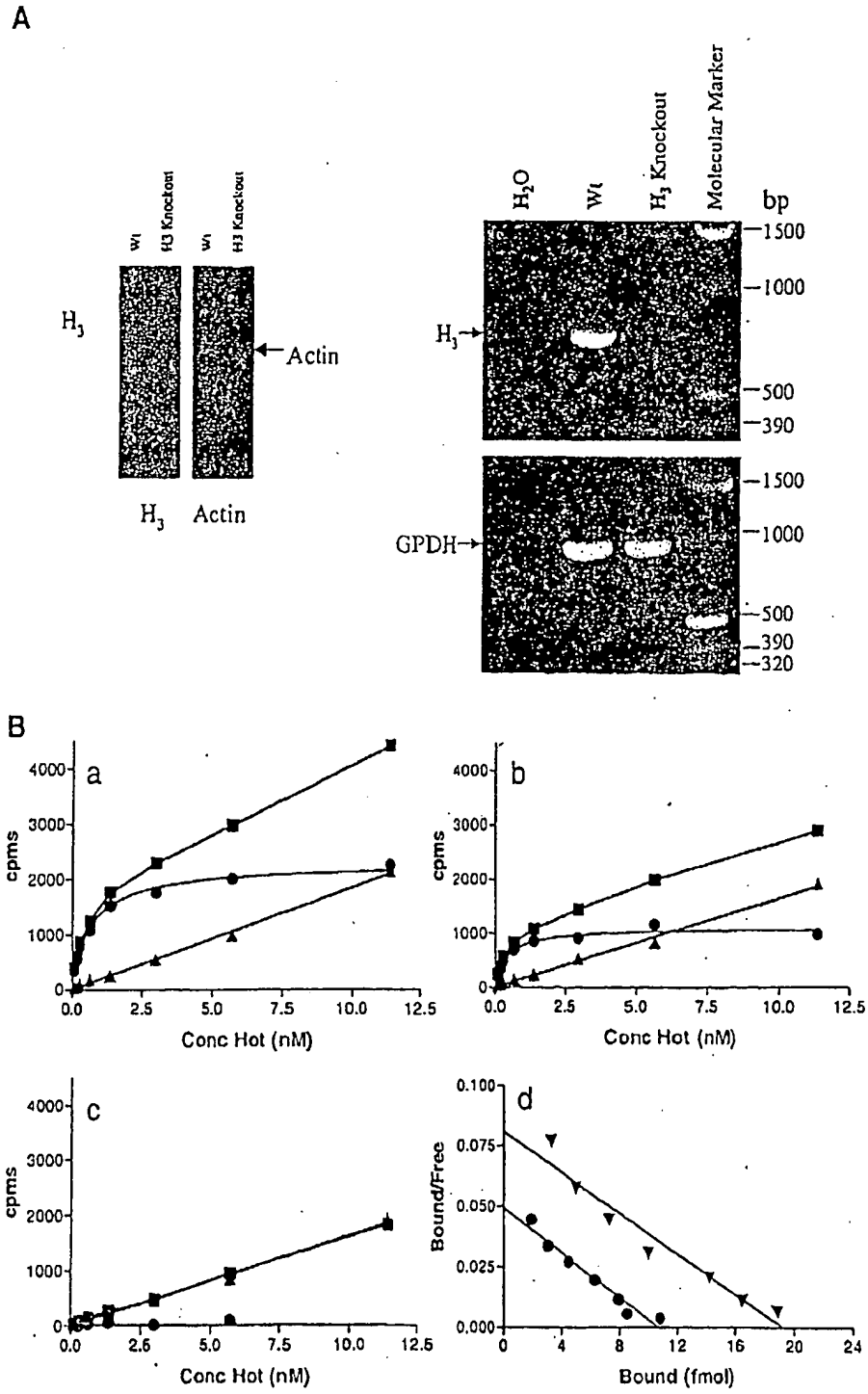

Fig. 1. Absence of histamine H_3 mRNA and (*R*)-[α-^{3}H] methylhistamine binding in brains from $H_3^{-/-}$ mice. A, left, northern blot. Total RNA (20 μg) from $H_3^{+/+}$ and $H_3^{-/-}$ mouse brains were transferred onto a Nytran nylon membrane and probed with a ^{32}P-labeled probe against mouse H_3 exon 3 DNA. As control, a mouse actin DNA probe was used in a parallel experiment. Right, reverse-transcriptase PCR. cDNA was synthesized from total RNA prepared from brains of $H_3^{+/+}$ and $H_3^{-/-}$ mice and used as template in a PCR reaction. The PCR products were run on a 1.5 % agarose gel and detected under UV light. As controls, mouse GDPH primers were used in a parallel PCR reaction. B, receptor binding of (*R*)-[α-^{3}H] methylhistamine to membranes prepared from $H_3^{-/-}$ (a), $H_3^{+/-}$ (b), or $H_3^{-/-}$ (c) mice. ■, total binding; ●, specific binding; ▲, nonspecific binding. Counts bound (cpms) are plotted on the *y*-axis, and the concentration of radiolabeled (*R*)-[α-^{3}H] methylhistamine is given on the *x*-axis. Whole brains (minus the cerebellum) were homogenized, and equal amounts of the resulting membrane preparation were included in each assay. d: Scatchard plots for the $H_3^{+/+}$ mice (▲) and $H_3^{-/-}$ mice (●).

treated group (Fig. 4B, left). We also tested the animals in the open field habituation test and found that the $H_3^{-/-}$out animals displayed normal learning. However, in contrast to the results in the passive avoidance test, the analgesia-inducing effect of scopolamine was not decreased in $H_3^{-/-}$ animals in the open field habituation test (not shown).

Discussion

In the central nervous system, histaminergic neurons are found only in the tuberomammillary nucleus of the posterior hypothalamus. These cells project to numerous brain regions (Brown et al., 2001) and are involved in many brain functions. The histamine H_3 receptor, a predominantly presynaptic inhibitory receptor, is involved in a number of physiological processes, including waking behavior and memory (Brown et al., 2001). The elucidation of its function has relied heavily on the use of H_3 receptor-specific ligands. The cloning of the H_3 receptor (Lovenberg et al., 1999) allowed a more molecular approach, including the production of knockout animals. In this report we describe some of the biochemical, behavioral, and pharmacological characteristics of the H_3 receptor knockout mouse.

We confirmed the absence of H_3 receptor in the brain of the knockout mice by RT-PCR, Northern blot, and binding studies. Because it has been suggested that the H_3 receptor may exist in several subtypes (West et al., 1990), binding studies using (*R*)-[α-^{3}H]methylhistamine, [*N*-α-^{3}H]methylhistamine, or [^{3}H]histamine were performed in mouse brain homogenates. No binding could be detected even at high (>100 nM) concentrations, which indicates that all H_3 binding is due to a single gene product and argues against the existence of H_3 receptor subtypes (data not shown). This also demonstrates that in whole brain homogenates, the recently identified H_4 receptor (Nakamura et al., 2000; Liu et al., 2001) does nc contribute to the observed binding of [^{3}H]histamine. This i consistent with the lack of apparent distribution of H_4 recep tor messenger RNA in brain tissue (Liu et al., 2001).

Histamine receptors play a complex role in the regulatio of brain levels of specific neurotransmitters. For instance, i H_1 receptor knockout mice, the serotonin turnover rate i increased in specific brain regions (Yanai et al., 1998). It i known that stimulation of the H_3 receptor increases th synthesis of histamine (Arrang et al., 1987b; Gomez-Ramire et al., 2002). Also, Yates et al. (1999) reported that thioper amide, a histamine H_3 receptor antagonist, enhanced th histamine turnover rate in rats. Therefore, we decided t measure brain levels of a number of neurotransmitters an their metabolites. The only change we found was a clea decrease in the levels of histamine in the cortex of $H_3^{-/}$ animals, which we attribute to the removal of the stimule tory effect of the H_3 receptor on the synthesis of histamine

The presynaptic autoregulatory H_3 receptor inhibits th neuronal release of histamine, which in turns leads to decreased stimulation of postsynaptic H_1 receptors (Brown e al., 2001). The role of the central H_1 receptor in arousal i extensively documented and underscored by the well know sedative effects of many H_1 receptor antagonists (Kay, 2000 Thus, the absence of H_3 receptors might be expected to pro mote histaminergic neurotransmission and thereby increas arousal in general. This was clearly not the case: the animal showed a decreased level of motor activity throughout th night (their active period) and decreased wheel running be havior that persisted during continuous darkness. There ar several possible explications for this. First, the histamine H receptor might be down-regulated in the $H_3^{-/-}$ mice to com pensate for the increased release of histamine at the synaps

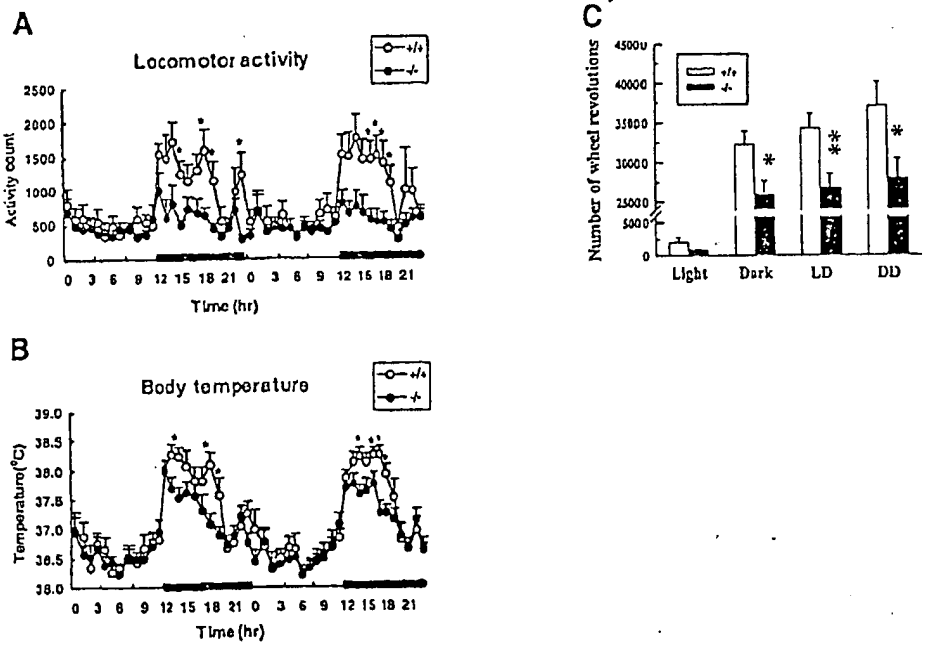

Fig. 2. Histamine H_3 receptor knockout mice have decreased locomotor activity (A), body temperature (B), and wheel running behavior (C). A and mean (±S.E.M.) total activity counts (A) and body temperature (B) over 48 h in $H_3^{+/+}$ (n = 10) and $H_3^{-/-}$ (n = 10) mice. Significant differences occurr between genotypes in the 12-h period of darkness (designated on the x-axis by black bars). Asterisks represent significant post hoc comparisons genotypes at individual 1-h intervals (*, $p < 0.05$). C, mean (±S.E.M.) wheel revolutions in $H_3^{+/+}$ (n = 8) and $H_3^{-/-}$ (n = 11) mice during a 24-h (12:12 LD) period (left three columns) and for a circadian cycle in constant darkness (DD) (right column). $H_3^{-/-}$ mice had significantly reduced wheel runnir activity during the LD (dark phase) and DD schedules compared with $H_3^{+/+}$ mice. *, p <0.05, *, p <0.01

We rejected this hypothesis after H_1 receptor binding experiments showed that $H_3^{-/-}$ mice had comparable densities of H_1 receptors compared with $H_3^{+/+}$ mice (data not shown). Second, the removal of the H_3 receptor may have led to perturbations in the homeostasis of neurotransmitter levels, an explanation that seems to be borne out by our results showing lower brain levels of histamine in $H_3^{-/-}$ animals. This leads to the interesting hypothesis that although the braking effect on histamine release exerted by the H_3 receptor is removed in the $H_3^{-/-}$ mice, a compensatory decrease in the availability of histamine in the nerve terminals may lead to an overall reduction of histaminergic neurotransmission with reduced stimulation of H_1 receptors and decreased locomotion as a consequence. This hypothesis could be approached by experiments measuring the release of histamine from brain slices derived from $H_3^{-/-}$ mice and by in vivo microdialysis. Alternatively, histaminergic neurons may have diminished histamine content as a result of the lack of inhibition of the H_3 receptor in its absence throughout the development of the animal. A careful study of developmental histamine turnover rates in H_3 receptor-deficient animals may help address this question.

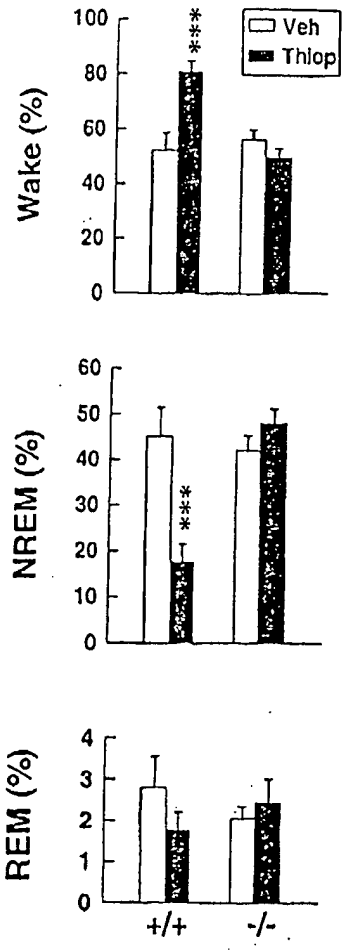

Fig. 3. H_3 receptor knockout animals are insensitive to the histamine H_3 receptor antagonist thioperamide. Mean (±S.E.M.) percentage of recording time spent in each of the three vigilance states (wake, NREM, and REM sleep) during the first 2 h of the light phase in both $H_3^{+/+}$ (n = 6) and $H_3^{-/-}$ (n = 7) mice. The open bars represent vehicle injections on baseline days, whereas the black bars represent thioperamide (10 mg/kg s.c.) injections given to the same animals the following day. Injections were performed at the onset of the light phase. ***, $p < 0.001$

Third, it must be pointed out that the influence of the histaminergic system on locomotor activity is complex. For instance, the H_1 knockout mice, which would be expected to show decreased locomotion throughout the light/dark cycle, actually displayed an increase in locomotor activity during the light phase (Inoue et al., 1996). In the present study, we saw no increase in activity during the light phase in $H_3^{-/-}$ mice, indicating that the components of the arousal system that are affected by knocking out the H_1 and H_3 receptors are different from one another. Finally, the mouse model described here is not a conditional knockout: the continuous absence of the H_3 receptor throughout the development of the animals may be compensated for by changes in other genes.

One of the most significant functions of the H_3 receptor seems to be its role in the regulation of waking behavior. Administration of H_3 receptor antagonists, such as thioperamide, increase wakefulness at the expense of REM and NREM sleep in rats (4 mg/kg i.p.) (Monti et al., 1991) and cats (2–10 mg/kg p.o.) (Lin et al., 1990). Thioperamide is a selective H_3 antagonist with a K_i of 4.2 nM at the rat H_3 receptor (Lovenberg et al., 2001). It is active in vivo at doses between 2 and 20 mg/kg i.p.(Stark et al., 1996). We therefore tested the effect of this compound (10 mg/kg s.c.) in $H_3^{-/-}$ mice. The $H_3^{+/+}$ animals responded to H_3 receptor blockade with a decrease in NREM sleep and increased waking, whereas the $H_3^{-/-}$ animals were completely insensitive to the wake-promoting effects of thioperamide. These experiments confirm the important role of the H_3 receptor as a mediator of wakefulness.

As mentioned earlier, the presynaptic H_3 receptor regulates the release of histamine and other neurotransmitters such as dopamine and acetylcholine (Hill et al., 1997). We therefore decided to investigate how these neurotransmitter systems were affected by ablation of the H_3 receptor. We probed dopaminergic neurotransmission by using methamphetamine, which increases locomotor activity and induces stereotypic behavior through an increase in dopamine release (Grilly and Loveland, 2001). We found that the $H_3^{-/-}$ mice scored lower on a stereotypy scale after methamphetamine administration than did the $H_3^{+/+}$ animals. The effect of methamphetamine on locomotor activity was less pronounced in the $H_3^{-/-}$ mice compared with $H_3^{+/+}$ animals as well. These results may indicate that the $H_3^{-/-}$ mice have slightly decreased dopaminergic activity, which may contribute to the overall spontaneous decrease in total activity and wheel running behavior observed in these animals. Our observations are in agreement with those of Clapham and Kilpatrick (1994), who found that the H_3 receptor antagonist thioperamide decreased amphetamine-induced locomotor activity in the mouse. However, because methamphetamine has several mechanisms of action, including serotonergic pathways, additional experiments with compounds selective for the various dopamine receptor subtypes will be needed to evaluate dopaminergic neurotransmission in more detail.

One of the most active areas of research in the H_3 receptor field is the study of memory. For instance, Molinengo et al. (1999) showed that the effects of thioperamide on memory consolidation seemed dependent on the situation, with or without painful stress. Also, Passani et al. (2000) indicated that thioperamide did not improve memory in normal mice, but only in a learning deficit situation. Because blockade of the presynaptic H_3 receptor leads to an increased release of

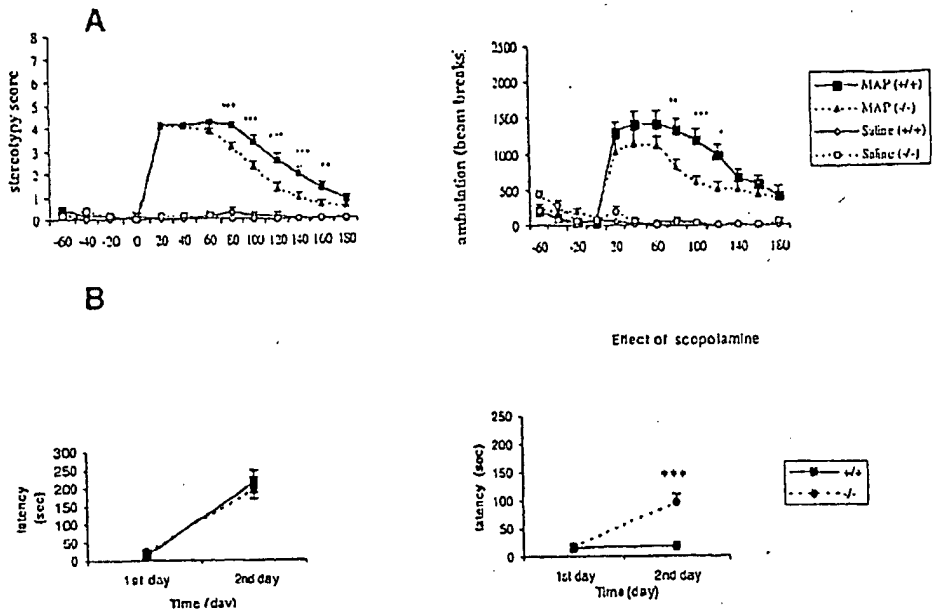

Fig. 4. H_3 receptor knockout mice exhibit decreased sensitivity to methamphetamine (A) and scopolamine (B). Means (±S.E.M.) of 7 saline-treated and 16 methamphetamine (MAP)-treated $H_3^{+/+}$ mice, and 8 saline-treated and 26 MAP-treated $H_3^{-/-}$ mice are shown. Data were collected from 60 min before and 180 min after injections. A, left, rating of stereotyped behavior in response to MAP (1 mg/kg) treatment. Right, ambulation of mice treated with saline or MAP (1 mg/kg). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. B, passive avoidance response. Left: mean (±S.E.M.) step-through latencies in untreated $H_3^{-/-}$ ($n = 13$) and $H_3^{-/-}$ ($n = 14$) mice. Right, effect of scopolamine (0.75 mg/kg) on step-through latencies in $H_3^{+/+}$ mice ($n = 14$) and $H_3^{-/-}$ mice ($n = 18$). The mice received scopolamine intraperitoneally 20 min before the first test in the passive avoidance chamber. No injection was given beforetesting on the second day. ***, $p < 0.001$

acetylcholine (Hill et al., 1997), models exploring the role of the cholinergic system in memory formation are particularly interesting. It is known, for instance, that the acetylcholine receptor antagonist, scopolamine, induces amnesia in the passive avoidance test and that H_3 receptor antagonists are able to prevent this effect (Giovannini et al., 1999). We found, using the passive-avoidance model, that $H_3^{-/-}$ mice showed normal learning behavior but were insensitive to the amnesia-inducing effects of scopolamine. This is consistent with previous reports showing that thioperamide could at least partially prevent the effects of scopolamine (Blandina et al., 1996; Onodera et al., 1998; Molinengo et al., 1999).

Interestingly, the $H_3^{-/-}$ mice were not insensitive to scopolamine in a second model, the open field habituation test. This test measures the habituation of exploratory activity and is a valid model of memory (Platel and Porsolt, 1982; Izquierdo et al., 1990). The fact that the $H_3^{-/-}$ mice were insensitive to the effect of scopolamine only when it was used in an aversion model may indicate a specific role of the H_3 receptor in the memory processes associated with painful stimuli.

Histamine, as a neurotransmitter, has been implicated in the regulation of many peripheral and CNS functions. However, the precise role that histamine and its receptors play in CNS physiology is not entirely clear. The present study in mice devoid of the H_3 receptor indicate an important role for the H_3 receptor in the regulation of locomotor activity and body temperature. In addition, $H_3^{-/-}$ mice are resistant to the amnesic effect of the cholinergic antagonist, scopolamine, and show a decreased response to the dopaminergic-stimulating drug, methamphetamine. Because the H_3 receptor has also been implicated in the regulation of a wide range of other physiological and behavioral processes, including food intake, digestion, cardiac and immune functions, cognition, and sleep, this new transgenic animal model should prove to be extremely important for elucidating the role of H_3 receptors in a variety of peripheral and CNS functions as well as pathophysiological states that are associated with altered histaminergic activity.

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Address correspondence to: Timothy W. Lovenberg, Johnson and Johnson Pharmaceutical Research and Development, LLC, 3210 Merryfield Row, San Diego CA 92121. E-mail: tlovenbe@prdus.jnj.com

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(71) Applicants: THE UNIVERSITY OF TOLEDO [US/US]; 2801 West Bancroft Street, Toledo, OH 43606 (US). GLIATECH, INC. [US/US]; 23420 Commerce Park Road, Beachwood, OH 44122 (US).

(72) Inventors: DURANT, Graham, J.; 55 Captain Luther Little Waye, Marshfield, MA 02050 (US). KHAN, Amin, M.; 6549 Park N, B-7, Solon, OH 44139 (US). TEDFORD, Clark, E.; 1448 Bell Road, S. Russell, OH 44022 (US).

(74) Agents: LAWRENCE, Stanton, T. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

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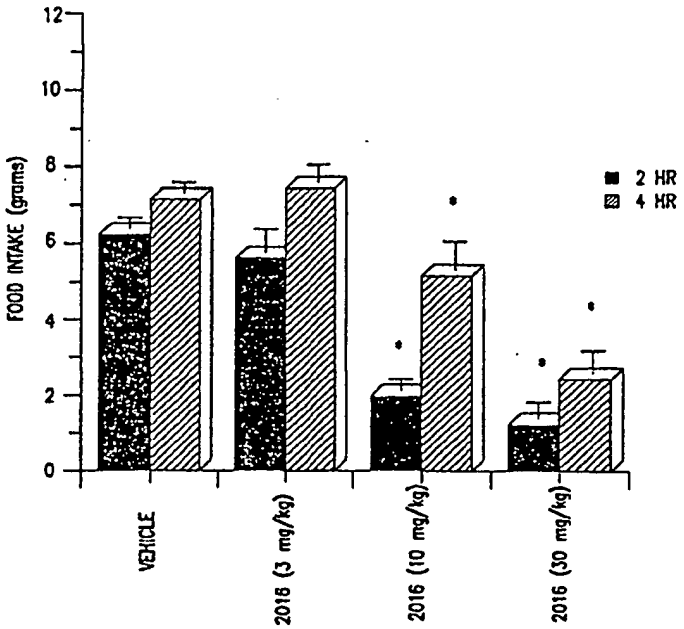
(54) Title: HISTAMINE H_3-RECEPTOR ANTAGONISTS AND THERAPEUTIC USES THEREOF



(57) Abstract

The present invention is directed to compounds of the class of piperidyl-imidazole derivative histamine H_3-receptor antagonist. Such compounds have affinity for histamine H_3-receptor, and preferably penetrate the blood-brain barrier. The compounds can block the soporific effect of an H_3-receptor agonist. Illustrative of the compounds of the invention is the molecule 4-(1-cyclohexylvaleroyl-4-piperidyl)-1H-imidazole. These compounds have been found to have utility in treating cognitive disorders and have been found to be useful as appetite suppressants.

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HISTAMINE H_3-RECEPTOR ANTAGONISTS AND THERAPEUTIC USES THEREOF

The present application is a continuation-in-part of International application No. PCT/US93/03104, filed March 31, 1993 which is a continuation-in-part of application Serial No. 07/862,657, filed April 1, 1992, each of which is incorporated herein by reference in its entirety.

1. FIELD OF THE INVENTION

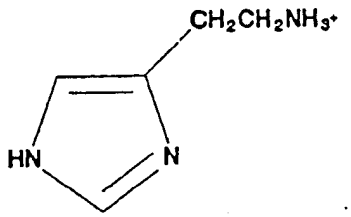
The present invention relates to novel compounds having potent activity as histamine H_3-receptor ("H_3") antagonists, and methods of using such compounds.

2. BACKGROUND OF THE INVENTION

Dementias tend to be characterized by cognitive disorders and often by depression. A particularly devastating dementia is Alzheimer's disease (AD). AD affects more than 30% of humans over 80 years of age, and as such, represents one of the most important health problems in developed countries (Evans et al., *J.A.M.A.* 262: 2551-2556 (1989); Katzman and Saitoh, *FASEB J.* 280: 278-286 (1991)). This neurodegenerative disorder of unknown etiology is clinically characterized by gradual impairment of cognitive function. The large buildup of intracytoplasmic neurofibrillary tangles and neurite plaques observed histopathologically in AD plausibly leads to degeneration of affected nerve cells. At least one study showed decreases in histamine and histidine levels in frontal, temporal and occipital cortices and in the caudate nucleus of brains from AD patients examined post mortem (Mazurkiewics and Wsonwah, *Can. J. Physiol. Pharmacol.*, 67:75-78 (1989)).

Histamine is a chemical messenger involved in various complex biological actions. It is widely distributed in the plant and animal kingdoms. In

mammals, including man, it occurs mainly in an inactive bound form in most body tissues. When released, histamine interacts with specific macromolecular receptors on the cell surface or within a target cell to elicit changes in many different bodily functions. Histamine (4(2-aminoethyl) imidazole) is a base. Its chemical structure is:



Histamine receptor pharmacology has revealed three subtypes of receptors which mediate (or are associated with) the activity of histamine. These receptors are most commonly referred to as H_1, H_2, and H_3. The most recently discovered of these receptors is the H_3 histamine receptor. Early studies suggested the presence of another histamine receptor when it was demonstrated that histamine inhibits its own synthesis and release in brain slices by a negative feedback process operating at the level of histaminergic nerve-endings (see, for example, Arrang, J.M. et al. *Nature* 302:832-837 (1983)). More recently, the H_3 receptor has been shown to function as a pre-synaptic autoreceptor inhibiting histamine synthesis and histamine release from neurons, especially in the control nervous system (Arrang, et al. *Nature* 327:117-123 (1987)). The presence of H_3 receptors in peripheral tissues has also been reported and here too they appear to be involved with the nervous system. Thus, histamine depresses sympathetic neurotransmission in the guinea pig mesenteric artery by interacting with H_3 receptors on the perivascular nerve

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terminals (Ishikawa and Sperelakis, *Nature* 327:158 (1987)). This important observation suggests that histamine may control the release of other neurotransmitters (Tamura et al., *Neuroscience* 25:171 (1988)). Inhibitory histamine H_3 receptors also exist in the guinea pig ileum where their role appears to be to modify the magnitude of histamine contraction, rather than affecting histamine release (Trzeciakowski, *J. Pharmacol. Exp. Therapy* 243:847 (1987)). Particularly intriguing is the discovery of H_3 receptors in the lung (Arrang et al. supra (1987)). The presence of histamine H_3 receptors in the lung raises the question of whether they control histamine release in anaphylaxis and whether they may be manipulated to provide therapy in asthma. Indeed it has been suggested that H_3 receptors may have a modulating role on excitatory neurotransmission in airways. Generally, however, H_3 receptor inhibition tends to increase histamine activity, with potentially detrimental effects. Thus, it is desirable to avoid introducing H_3 receptor antagonists that act on peripheral tissues.

Histamine H_3 receptor activation was found to inhibit acetylcholine release in a guinea pig ileum model (Poli et al., *Agents and Actions* 33: 167-169). Selective H_3-receptor blockers reversed the histamine-induced inhibitory effect. Histamine also decreased serotonin release; this effect was reversed with an H_3-antagonist, and was suggested to operate via the histamine H_3-receptors (Schlicker et al., *Naunyn-Schmiedaberg's Arch. Pharmacal.* 337: 588-590 (1988). Activation of H_3-receptors was found to inhibit excitatory presynaptic potentials (Arrang et al., *J. Neurochem.* 51:105 (1988)).

One reported highly specific competitive antagonist of histamine H_3 receptors is thioperamide (Arrang et al., supra (1987)). Although thioperamide is a very potent antagonist *in vitro* (K_i = 4.3 nmol/L), relatively high doses are required *in vivo* to inhibit histamine release from the brain in rats (Ganellin et al., *Collect. Czech. Chem. Commun.* 56:2448-2455 (1991)). Ganellin et al. suggests that this most probably results from poor penetration through the blood-brain-barrier by this peramide, although the pharmacokinetic properties of thioperamide may also play a role. Moreover, the thiourea functionality found in thioperamide may result in higher intrinsic toxicity of thioperamide.

Thiourea-containing drugs are known to be associated with undesirable side effects in clinical use. For example, with thiourea-containing drug molecules that are used to treat hyperthyroidism, agranulocytosis is known to be a serious, and occasionally fatal, toxic effect in clinical use (see, e.g., Brimblecombe et al. *Gastroenterology* 74:339-346 (1978)). The thiourea-containing histamine H_2-receptor antagonist metiamide caused a low incidence of granulocytopenia in peptic ulcer patients and was withdrawn from clinical use (Forrest et al., *Lancet* 1: 392-393 (1975)). In high dose, repeated dose toxicological studies in dogs, incidences of agranulocytosis were seen at 162 mg/kg/day (Brimblecombe et al., "Toxicology of Metiamide," *International Symposium on Histamine H_2 - Receptor Antagonists*, Wood and Simpkins, Smith Kline & French, pp. 53-72 (1973)). A proportion of dogs (<10%) died acutely with pulmonary edema and pleural effusion. The metiamide isostere cimetidine, in which the thiourea group was replaced by an alternative group

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(cyanoguanidine), did not cause granulocytopenia, or any other side effects in animal toxicity studies or in clinical usage by multimillions of patients, indicating that the toxicological problems with metiamide could be attributed to the presence of the thiourea group (Brimblecomb et al., *supra*). It is likely that the thiourea functionality, with its association with toxicological phenomena and its likelihood of inducing undesirable side effects, could limit the clinical development of thioperamide.

Although some predictions have been made concerning the ability of molecules to pass through the blood brain barrier, these predictions are at best speculative. The rate and extent of entry of a compound into the brain are generally considered to be determined primarily by partition coefficient, ionization constant(s) and molecular size. No single partition solvent system has emerged as a universally applicable model for brain penetration, although the octanol water system has received particular attention, and Hansch and coworkers have suggested that a partition coefficient in this system of about 100 is optimal for entry into the central nervous system (CNS) (Glave and Hansch, *J. Pharm. Sci.*, 61:589 (1972); Hansch et al., *J. Pharm. Sci.*, 76:663 (1987)). Comparisons between known H_2 antagonists, however, suggest that there is no such simple relationship between their brain penetration and octanol water partition coefficients (Young et al., *J. Med. Chem.* 31:656 (1988)). The comparison of the ability of histamine H_2 receptor antagonists to cross the blood brain barrier suggests that brain penetration may increase with decreasing over-all hydrogen binding ability of a compound (Young et al., supra). However, optimizing H_2 receptor antagonists to improve brain

penetration reduced antagonist potency (Young et al., supra). Thus it is fundamentally difficult to optimize both blood brain barrier permeability and function of a compound.

It is therefore an object of the present invention to provide novel potent histamine H_3-receptor antagonists that are better able to penetrate the blood-brain-barrier than previously reported compounds.

Further it is an object of the present invention to provide novel potent histamine H_3-receptor antagonists that have reduced toxicity compared to other known H_3 antagonists.

Another object of the present invention is to provide histamine H_3-receptor antagonists that will act selectively on the brain and have limited activity on H_3 receptors in peripheral tissues.

It is yet another object of the present invention to provide a novel class of histamine H_3-receptor antagonists.

3. SUMMARY OF THE INVENTION

The present invention provides novel compounds having activity as histamine H_3-receptor antagonists. In a preferred aspect, the compounds of the invention exhibit ready penetration of the blood-brain-barrier and reduced toxicity. The novel compounds of this invention include compounds of the formula:

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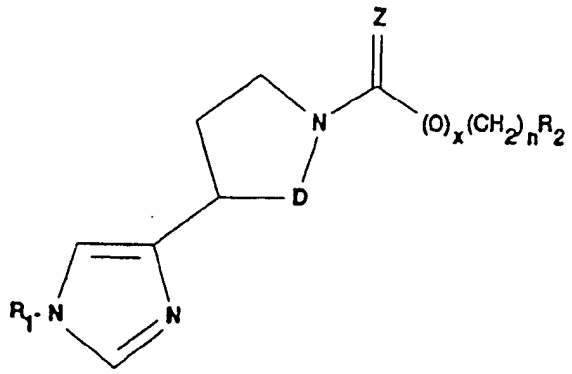

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wherein D is CH_2 or CH_2-CH_2, Z represents S or O, preferably O, x is 0 or 1, n is an integer from 0 to 6, R_1 represents preferably hydrogen, or a hydrolyzable group, but can be a lower alkyl or aryl group, and R_2 represents a linear chain, branched chain or carbocyclic group or aryl group of up to about 20 carbon atoms, and salts thereof. If R_2 is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0. If R_2 is adamantane, the sum of x and n must be greater than 1. The various alkyl or aryl groups can have functional group substituents.

It has been discovered that amide or carbamate functional groups can be used to join alkyl or aryl substituents to the piperidyl nitrogen of 4(4-piperidyl)-1H-imidazole groups. Other cyclic imides, particularly pyrrolidyl or cycloheptamidyl ($C_6H_{11}N$) can be substituted for piperidine. In a preferred aspect, the compounds of the invention are surprisingly effective at transport across the blood brain barrier, thus limiting their effects primarily to cerebral histamine H_3-receptors, and are also less toxic than histamine H_3-receptor antagonists based on a thiourea functional group.

In addition, the present invention encompasses a pharmaceutical composition comprising a compound of

the invention, and a method of using a compound or pharmaceutical composition of the inspection in an animal, particularly in a human, to treat Alzheimer's disease and other dementias by ameliorating the cognitive defects and neurodegenerative effects associated therewith. The histamine H_3-receptor antagonists of the invention have additional therapeutic uses where increased arousal and attention is desired.

Furthermore, the present invention encompasses a method of suppressing appetite in an animal, particularly a human, comprising administering to the animal an effective amount of a compound of the present invention. Thus, the invention encompasses the treatment of obesity in animals suffering from such a disorder. The invention further encompasses a pharmaceutical composition comprising a compound of the present invention; and optionally a pharmaceutically acceptable carrier. This pharmaceutical composition can be administered to suppress appetite in an animal, particularly a human.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Binding of N^{α}-methylhistamine to rat cortical homogenate. Open box: total bound; x'ed box: specific binding; closed box: non-specific binding.

FIG. 2. Binding of ^3H-labeled N^{α}-methylhistamine to the cortical homogenate of thioperamide injected rats.

FIG. 3. Binding of ^3H-labeled N^{α}-methylhistamine to the cortical homogenate of compound 1 injected rats.

FIG. 4. The effect of α-methylhistamine on sleeping one hour after injection.

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FIG. 5. The effect (dose-response) of thioperamide on sleep induced by R(-)-α-methylhistamine (30 mg/kg).

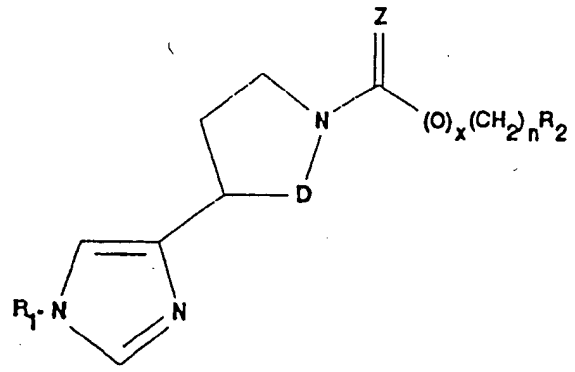
FIG. 6. The effect (dose-response) of compound 1 on sleep induced by R(-)-α-methylhistamine (25 mg/kg).

FIG. 7. The effect of compound 1 on 2 and 4 hour food intake in 24-hour-fasted rats. Solid bar: 2 hour food intake. Cross-hatched bar: 4 hour food intake.

FIG. 8. The effect of compound 1 on 6 hour food intake in 24-hour-fasted rats.

5. DETAILED DESCRIPTION OF THE INVENTION

The compounds of the present invention are compounds of the general formula.



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wherein D is CH_2 or CH_2-CH_2, Z represents sulfur (S) or oxygen (O), preferably O, x is 0 or 1, n is an integer from 0 to 6, R_1 represents hydrogen, an *in vivo* hydrolyzable group, a lower alkyl group, a lower cyclic alkyl group, or a lower aryl group, and R_2 represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 carbon atoms including mono and bicylic moieties, and a substituted or an

unsubstituted aryl group of up to about 20 carbon atoms, or any combination of above-mentioned groups, or salts thereof. In a specific embodiment, R_2 can represent a disubstituted methyl, such as but not limited to dicyclohexyl methyl ($-CH(C_6H_{11})_2$), diphenyl methyl ($-CH(C_6H_5)_2$), and the like. If R_2 is tert-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0. If R_2 is adamantane, the sum of x and n must be greater than 1.

In a preferred embodiment, R_1 is hydrogen. It is also contemplated that R_1 can be a hydrolyzable leaving group, such as an acyl or carbamyl, including where $R_1=-CZ(O)_x(CH_2)_nR_2$, as in I above. It is well known that N-acylimidazoles are hydrolytically labile, and R_1 may be selected such that it yields the parent imidazole compound *in vivo* at an optimal rate. Such hydrolysis will yield the compound with hydrogen as R_1. Thus, the contemplated compounds of the invention with a hydrolyzable substituent at R_1 are functionally equivalent to the preferred embodiment, i.e., where R_1 is hydrogen. R_1 can also be a lower linear chain, branched chain, or cyclic alkyl, or a lower aryl. The term "lower" as applied to the alkyl or aryl substituents at R_1 indicates the presence of up to seven carbon atoms. In specific embodiments infra, R_1 is methyl, benzyl, methylcyclohexane, N-cyclohexylformamide, benzaldehyde, and t-butylaldehyde.

In yet a further embodiment, the nitrogen atom at position 3 of the imidazole ring can be substituted with a lower alkyl or aryl group, or with a hydrolyzable leaving group.

In a preferred embodiment, D is CH_2-CH_2, resulting in a piperidine ring structure. However, it is contemplated that D can be CH_2, yielding a pyrrolidine

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ring structure. In yet another embodiment, D can be $(CH_2)_3$, yielding a cycloheptimide (seven membered heterocycle with one nitrogen). While orientation of the imidazole group distal to the N of the piperidine is preferred, the invention contemplates the imidazole at the 2 or 3 position on the piperidine (or the 2 position of pyrrolidine, or the 2 and 3 position of the cycloheptimide ring). These alternate embodiments can be used instead of the piperidyl embodiment with the imidazole group located at the 4 position, although the piperidyl embodiment is preferred.

Although the present invention is not limited to any mechanistic theory, it is believed that the blood brain barrier is permeable to the compounds of the present invention in part because of the subtle decrease in polarity afforded by an amide or carbamate bond linking the $(-(O)_x(CH_2)_nR)$ moiety (e.g., a hydrophobic tail) to the 4(4-piperidyl)-1H-imidazole (or 4(3-pyrrolidyl)-1H-imidazole) structure. With slightly less polarity and hydrogen-bonding capability than urea or thiourea, the amide or carbamate functionality can more efficiently traverse the blood brain barrier. Moreover, the dipole of the amide or carbamate is distal to the hydrophobic tail, more proximal to the imidazole (which is a fairly polar group), and thus tends to effect greater amphiphilicty in the molecule. That the compounds of the invention retain amphiphilic character is important for solubility in aqueous solution. Solubility in aqueous solution is desirable for a compound to be used therapeutically in an animal particularly in a human. That such a subtle difference, use of an amide or carbamate functionality, should perceptibly alter blood brain barrier permeability may be considered to be surprising since it is not generally appreciated.

In preferred embodiments, a bulky hydrocarbon R_2 group is chosen so that the net hydrophilicity of the H_3-receptor antagonist is increased, and the steric effects of a bulky substituent at R_2 are decreased, by increasing the number of methylenes in a straight chain alkyl group (i.e., in Formula I, $n > 1$). In a specific embodiment, a tetramethylene bound to the amide or carbamate group is used. Preferably a cyclic alkyl or aryl group is linked to the amide or carbamate via the straight chain alkyl group. In a specific embodiment, tetramethylene cyclohexane (cyclohexylbutyl) is bound to an amide. Although specific hydrophobic alkyl and aryl groups have been mentioned, one of ordinary skill in the art will recognize that there are many possible hydrophobic groups for use in the compounds of the invention. These fall within the scope of the instant invention.

Thus, R_2 can be one or more bulky substituent groups. As stated above, in a preferred aspect of the invention, the bulky substituents are removed from the amide or carbanate group on the piperidyl-imidazole by increasing n. In one embodiment, R_2 is CHR_3R_4, in which n is 3 or 4 and R_3 and R_4 are cyclohexyl, phenyl, or the like. R_3 and R_4 can be the same group or different groups. In another embodiment, R_2 is decalin or adamantane or the like. If R_2 is adamantane, preferably n is greater than 1, but the sum of x and n must be greater than 1.

As used herein, the phrase linear chain or branched chained alkyl groups of up to about 20 carbon atoms means any substituted or unsubstituted acyclic carbon-containing compounds, including alkanes, alkenes and alkynes. Examples of alkyl groups include lower alkyl, for example, methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl or tert-butyl; upper

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alkyl, for example, octyl, nonyl, decyl, and the like; and lower alkylene, for example, ethylene, propylene, propyldiene, butylene, butyldiene, and the like. The ordinary skilled artisan is familiar with numerous linear and branched alkyl groups, which are with the scope of the present invention.

In addition, such alkyl group may also contain various substituents in which one or more hydrogen atoms has been replaced by a functional group. Functional groups include but are not limited to hydroxyl, amino, carboxyl, amide, esther, ether, and halogen (fluorine, chlorine, bromine and iodine), to mention but a few.

As used herein, substituted and unsubstituted carbocyclic groups of up to about 20 carbon atoms means cyclic carbon-containing compounds, including but not limited to cyclopentyl, cyclohexyl, cycloheptyl, admantyl, and the like. Such cyclic groups may also contain various substituents in which one or more hydrogen atoms has been replaced by a functional group. Such functional groups include those described above, and lower alkyl groups as described above. The cyclic groups of the invention may further comprise a heteroatom. For example, in a specific embodiment, R_2 is cyclohexanol.

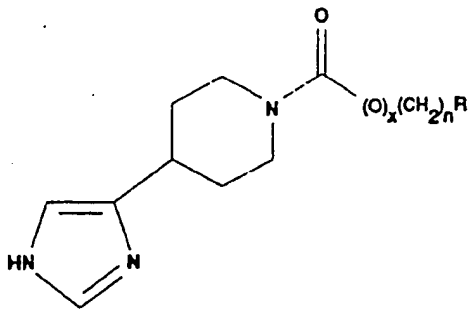
As used herein, substituted and unsubstituted aryl groups means a hydrocarbon ring bearing a system of conjugated double bonds, usually comprising six or more even number of π (pi) electrons. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, anisyl, toluyl, xylenyl and the like. According to the present invention, aryl also includes heteroaryl groups, e.g., pyrimidine or thiophene. These aryl groups may also be substituted with any number of a variety of functional groups. In addition

to the functional groups described above in connection with substituted alkyl groups and carbocyclic groups, functional groups on the aryl groups can be nitro groups.

As mentioned above, R_2 can also represent any combination of alkyl, carbocyclic or aryl groups, for example, 1-cyclohexylpropyl, benzyl cyclohexylmethyl, 2-cyclohexylpropyl, 2,2-methylcyclohexylpropyl, 2,2-methylphenylpropyl, 2,2-methylphenylbutyl.

In a specific embodiment, R_2 represents cyclohexane, and n=4 (cyclohexylvaleroyl). In another specific embodiment, R_2 represents cinnamoyl.

Particularly preferred are compounds of the formula:



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wherein x is 0 or 1, n is an integer from 0 to 6, more preferably n = 3-6, and most preferably n=4, and R is as defined for R_2 above. Examples of preferred alkyl groups for R include but are not limited to cyclopentyl, cyclohexyl, admantane methylene, dicyclohexyl methyl, decanyl and t-butyryl and the like. Examples of preferred aryl and substituted aryl groups include but are not limited to phenyl, aryl cyclohexyl methyl and the like.

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5.1. SYNTHESIS OF THE COMPOUNDS

The compounds of the present invention can be synthesized by many routes. It is well known in the art of organic synthesis that many different synthetic protocols can be used to prepare a given compound. Different routes can involve more or less expensive reagents, easier or more difficult separation or purification procedures, straightforward or cumbersome scale-up, and higher or lower yield. The skilled synthetic organic chemist knows well how to balance the competing characteristics of synthetic strategies. Thus the compounds of the present invention are not limited by the choice of synthetic strategy, and any synthetic strategy that yields the compounds described above can be used.

As shown in the Examples, *infra*, two general procedures can be used to prepare the instant compounds. Both involve condensation of an activated (electrophilic) carbonyl with the nucleophilic piperidyl nitrogen of 4-(4-piperidyl)-1H-imidazole.

The first procedure involves preparing the acid chloride derivative or acid anhydride of a carbonyl, i.e., activating the carbonyl. This activated carbonyl is added in molar excess to the piperidyl-imidazole in the presence of a molar excess of an unreactive base, for example, but not limited to, dicyclohexyl amine.

The second procedure is to condense the piperidyl-imidazole with a slight molar excess of a dicarbonate, again in the presence of an unreactive base, for example and not by way of limitation, triethylamine. This method can be used especially in the preparation of carbamate compounds.

A preferred synthesis of the 4-(4-piperidyl)-1H-imidazole is also provided. Commercially available 4-

acetyl pyridine (Aldrich Chemical Co.) is converted into the key intermediate 4-(4-pyridyl)-1H-imidazole by bromination with hydrogen bromide in acetic acid (Barlin, et al., *Aust. J. Chem.* 42:735 (1989)) to yield the bromoacetyl pyridine in high yield. Reaction of bromoacetyl pyridine with formamide at 110°C affords the substituted imidazole in high yield. The reaction is usually performed without the addition of any solvent. The pyridyl moiety is reduced by catalytic hydrogenation using 5-10% Rhodium on carbon in acidified water at a pressure of 20-55 atmospheres to yield 4-(4-piperidyl)-1H-imidazole. This synthesis is disclosed more fully in copending United States patent application Serial No. 07/862,658, filed by the instant inventors on April 1, 1992, entitled "PROCESS FOR THE PREPARATION OF INTERMEDIATES USEFUL FOR THE SYNTHESIS OF HISTAMINE RECEPTOR ANTAGONISTS," which is specifically incorporated herein by reference in its entirety.

Solvents for use in the synthesis of the compounds of the invention are well known in the art. The solvent must be non-reactive, and the starting materials and base must be soluble in the solvent. Preferably, an aprotic organic solvent of medium to high polarity is used. For example, acetonitrile, can be used. Under appropriate conditions, in the synthesis of carbamates of the invention, an alcohol, e.g., methanol, can be used.

The electrophilic carbonyl group, which contains the R_2 moiety, can be obtained from commercial sources, or it may be prepared synthetically. In specific examples, *infra*, the carbonyl is obtained commercially. Activation of carbonyls is well known. The acid chloride can be prepared by reacting the carboxylic acid with sulfonyl chloride.

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Alternatively, the acid chloride may be available commercially. In specific embodiments, *infra*, acid chlorides were obtained from commercial sources (Aldrich Chemical). Similarly, the acid anhydride can be prepared conveniently by reaction of a salt of the carboxylic acid with the acid chloride. In a specific embodiment, the carboxylic acid is reacted with a carbonate acid chloride to form an asymmetric acid anhydride. In another embodiment, the acid anhydride can be obtained commercially. In a specific embodiment, *infra*, the acid anhydride was obtained from Aldrich Chemical. Dicarbonates for use in the invention are available commercially, e.g., from Aldrich Chemical.

5.2. BIOLOGICAL ACTIVITY

The compounds of the present invention are biologically active in assays for histamine H_3-receptor antagonist activity, as well as in a radioligand binding assay in rat brain membranes (e.g., Table I, *infra*). The binding assay procedure used and its standardization with known H_3-receptor antagonists is shown in the examples *infra*.

Further biological studies can demonstrate that the histamine H_3-receptor antagonists of this invention reverse the soporific effects of the histamine H_3-receptor agonist, R(-)-alphamethylhistamine in mice when both drugs are administered peripherally *(infra)*. In a specific embodiment, the compound designated No. 2016 reverses the soporific effect of R(-)-alphamethylhistamine.

The data in the Examples, *infra*, support the view that antagonists of histamine H_3-receptors of the invention are useful regulators of the sleep-

wakefulness cycle with potentially useful cognitive and behavioral effects in mammals including humans.

In vivo studies can be used to show effectiveness of a compound of the invention to cross the blood-brain barrier, as shown in the examples, *infra*. The data support the view that drugs of the present invention penetrate the blood brain barrier and are able to exert beneficial central actions in mammals when these drugs are administered to the peripheral circulation.

5.3. THERAPY

The histamine H_3-receptor antagonists of the invention can be provided therapeutically for the treatment of a subject suffering from a cognitive disorder or an attention or arousal deficit, according to the present invention. One of ordinary skill in the art would readily determine a therapeutically effective dose of an H_3 receptor antagonist of the invention based on routine pharmacological testing and standard dosage testing. In one aspect of the present invention, the compounds can be administered in doses of about 0.01 to about 200 mg/kg, more preferably 1 to 100 mg/kg, and even more preferably 30 to 100 mg/kg. In a specific embodiment, greater than about 20 mg/kg of a compound of the invention was effective to reduce the soporific effect of (R)α-methylhistamine. Included in the routine pharmacological testing are toxicity studies to determine an upper limit dose. Such toxicity studies can include LD_{50} studies in mice, and 15 day toxicity studies in mammals.

The histamine H_3-receptor antagonists of the invention are believed to increase the release of cerebral histamine, acetylcholine and serotonin. These compounds can lead to increased arousal and

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attention. They can also be of benefit in the treatment of cognitive disorders.

Therapy with a compound of the invention is indicated to treat dementia, as either a primary or an adjunct therapy. The compounds of the invention have clinical utility in the treatment of dementia disorders in general. In a preferred embodiment, a compound of the invention can be used in the treatment for Alzheimer's disease. The compounds can also be used to treat presenile and senile dementia, Huntington's chorea, tardive dyskinesia, hyperkinesia, mania, Tourette syndrome and Parkinson's disease, to name but a few. Other specific indications include the treatment of narcolepsy and hyperactivity in children. In another embodiment, the compounds of the invention can be used in the treatment of certain psychoses, for example forms of depression or schizophrenia.

The compounds of the invention can be used to arouse victims of comas induced by stroke, drugs or alcohol. In another embodiment, the compounds of the invention can be used to increase wakefulness, where this effect is desired. For example, the compounds of the invention, which are preferentially targeted to H_3 receptors in the brain, can be used to counteract the soporific effect of some antihistamines without negating the therapeutic effects of the antihistamines on peripheral tissue, e.g., lung. Thus allergy patients can relieve some of the side effects of antihistamine therapy. Similarly, the compounds of the invention can be used to reverse overdose of barbiturates and other drugs.

5.3.1. Appetite Suppression

The histamine H_3-receptor antagonists of the present invention have been found to be useful in animals, particularly humans, as appetite suppressants. Thus, in another embodiment of the present invention, these compounds are used to control weight gain, to treat obesity and to promote weight loss, as well as any other condition wherein appetite suppression is desirable.

The present invention provides a method of suppressing appetite in an animal, including but not limited to a human, which comprises administering to the animal, an effective amount of a compound of the present invention.

In specific embodiments, the present invention encompasses a method of suppressing appetite in an animal, including but not limited to a human, which comprises administering to an animal, including a human, an effective amount of a compound of formula I or formula II, or a pharmaceutically acceptable salt thereof.

In a most preferred embodiment, the present invention encompasses a method for suppressing appetite in an animal, including but not limited to a human, which comprises administering to an animal, including a human, an effective amount of 4-(1-cyclohexylvaleroyl-4-piperidyl)-1H-imidazole (compound 1) or a pharmaceutically acceptable salt thereof.

The invention also provides pharmaceutical compositions useful as appetite suppressants which comprise an effective amount of a compound of the present invention or a pharmaceutically acceptable salt thereof; and optionally a pharmaceutically acceptable carrier or excipient. In particular, the present invention encompasses a pharmaceutical

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composition comprising an effective amount of 4-(1-cyclohexylvaleroyl-4-piperidyl)-1H-imidazole (compound 1) or a pharmaceutically acceptable salt thereof; and optionally a pharmaceutically acceptable carrier or excipient.

An effective amount is an amount sufficient to achieve appetite suppression. One of ordinary skill in the art can readily determine an effective amount of a compound of the present invention based on routine pharmacological testing and standard dosage testing. In one embodiment of the present invention, an effective amount of the compounds disclosed here is about 0.01 to about 200 mg/kg, more preferably 0.1 to 100 mg/kg, and even more preferably 30 to 100 mg/kg. The doses of the present invention can be given in divided or multiple doses over time as needed by the particular subject.

It is noted that the terms "appetite suppression" or "suppressing appetite" are known to those skilled in the art and these are used herein consistently therewith. These terms as used herein include a reduction, decrease or amelioration in appetite, a reduction, decrease or amelioration in the desire or craving for food, a reduction, decrease or amelioration in food intake. Appetite suppression can result in weight loss or weight control as desired. In a specific embodiment, the appetite suppressant of the present invention can be used to treat obesity and any related condition in which weight control is desired, including but not limited to severe and moderate obesity as well as overweight subjects.

5.4. PHARMACEUTICAL COMPOSITIONS AND METHODS OF ADMINISTRATION

The effective dose of a compound of the invention, and the appropriate treatment regime can

vary with the indication and patient condition, e.g., the treatment of a dementia or the treatment of tiredness may require different doses and regimens. These parameters are readily addressed by one of ordinary skill in the art and can be determined by routine experimentation.

A therapeutic compound of the invention may also contain an appropriate pharmaceutically acceptable carrier or excipient, diluent or adjuvant, i.e., the compound can be prepared as a pharmaceutical composition. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. When administering the compounds of the present invention as an appetite suppressant, the preferred dosage form is orally by tablet, pill or capsule. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain an effective therapeutic amount of the active compound together with a suitable

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amount of carrier so as to provide the form for proper administration to the patient. While intravenous injection is a very effective form of administration, other modes can be employed, including but not limited to intraventricular, intramuscular, intraperitoneal, intra-arteriolar, and subcutaneous injection, and oral, transdermal, nasal and parenteral administration.

The therapeutic agents of the instant invention may be used for the treatment of animals, and more preferably, mammals, including humans, as well as mammals such as dogs, cats, horses, cows, pigs, guinea pigs, mice and rats.

In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann.*

Neurol. 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

6. EXAMPLES

A series of compounds were prepared and tested for their histamine H_3 receptor antagonist activity. The results are summarized in Table 1. The antagonist activity of the compounds was detected by observing inhibition of (^{3}H)-N-(alpha)methylhistamine activity on rat brain membranes.

6.1. SYNTHESIS OF THE COMPOUNDS

The amide and carbamate compounds of Table 1 were synthesized from 4-(4-piperidyl)-1H-imidazole by three general procedures:

Procedure A: 4-(4-piperidyl)-1H-imidazole and the appropriate acid chloride were conjugated using dicyclohexylamine as base according to the following scheme:

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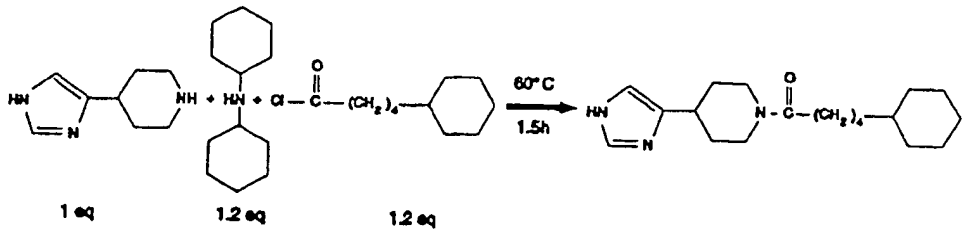
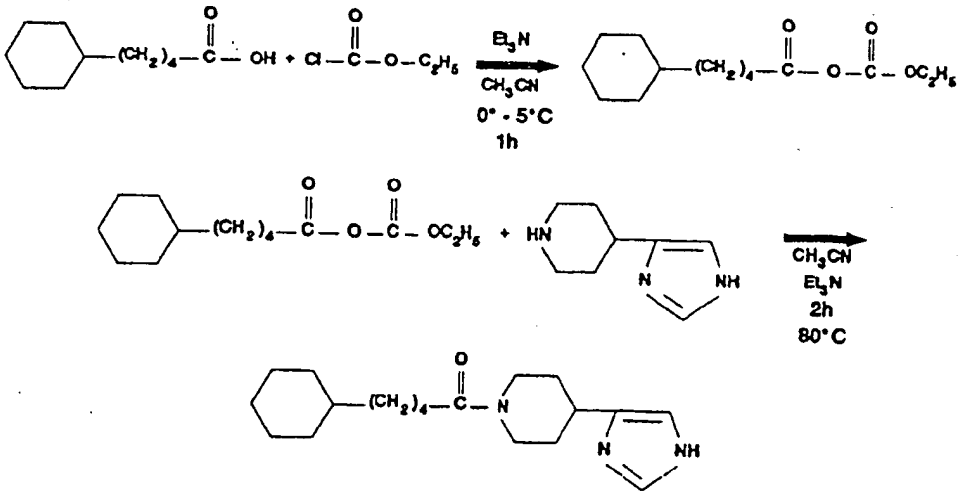
Scheme I



Procedure B: 4-(4-piperidyl)-1H-imidazole and the corresponding acid anhydride were conjugated using triethylamine as base according to the following scheme:

Scheme II

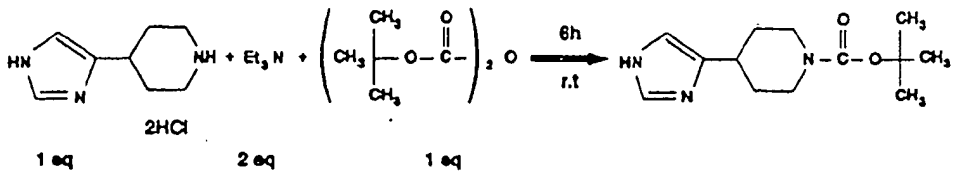


Procedure C: 4-(4-piperidyl)-1H-imidazole and the corresponding dicarbonate were conjugated using triethyl amine as a base according to the following scheme:

6.1.1. PREPARATION OF 4-(1-CYCLOHEXYLVALEROYL-4-PIPERIDYL) 1H-IMIDAZOLE (COMPOUND 1)

To a mixture of 755 mg (5.00 mmol) 4-(4-piperidyl)1-H-imidazole and 942 mg (5.20 mmol) of

Scheme III



dicyclohexylamine in 10 ml anhydrous acetonitrile at 25°C was slowly added 1.06 g (5.20 mmol) cyclohexanevaleroyl chloride in 2 ml of dichloromethane over a period of 10 min with stirring; then the reaction mixture was heated at 60°C for 1.5 h. After cooling to ambient temperature, the solid side product that was obtained (dicyclohexylammonium chloride) was filtered off and the filtrate was concentrated in vacuo to remove acetonitrile. The resulting crude oil was crystallized with methanol: anhydrous diethyl ether to give 1.085 mg of analytically pure product as a yellow powder. Yield: 68%; M.P.: 159°C; MS: m/e=317(M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.65 (s, 1H), 6.75 (s, 1H); cyclohexylbutyl: δ 2.20 (m, 8H), 1.20 (m, 11H); piperidyl: 4.65 (d, 2H), 3.95 (d, 2H), 3.10 (d, 2H), 2.84 (m, 1H), 2.20 (m, 2H).

Compounds No. 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 in Table I were synthesized in similar manner, i.e., by condensation of the acid chloride with 4(4-piperidyl) 1-H-imidazole in the presence of dicyclohexylcarbodiimide. Purified product was obtained by preparative TLC Silica Gel GF. 60 (2000 Microns) and the solvent of recrystallization was methanol:anhydrous ether (20:80).

Compound No. 3, yield: 70%; oil; MS m/e 275 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.60 and 6.75 (s, 1H); piperidine H: complex, δ 4.65 (d, 2H), 3.90 (d, 2H),

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3.10 (m, 3H), 2.10 (m, 2H); cyclohexyl acetyl H: δ 1.50 (m, 11H), 2.80 (m, 2H).

Compound No. 4, yield: 67%; oil; MS: m/e 267 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.50 and 6.60 (s, 1H); piperidine H: complex, δ 3.90 (d, 2H), 2.80 (m, 3H), 2.55 (m, 2H), 1.80 (m, 2H); phenyl acetyl H: δ 7.10 (m, 5H), 1.50 (m, 2H).

Compound No. 5, yield: 71%; oil; MS: m/e 297 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.80 and 6.70 (s, 1H); piperidine H: complex, δ 4.60 (d, 2H), 3.80 (d, 2H), 3.10 (m, 3H), 1.80 (d, 2H); phenyl propyl H: δ 7.20 (m, 5H), 2,65 (m, 2H), 235 (m, 2H), 2.10 (m, 2H).

Compound No. 6, yield: 74%; oil; MS: m/e 289 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.70 and 6.80 (s, 1H); piperidine H: complex, δ 4.60 (d, 2H), 3.85 (d, 2H), 3.10 (m, 3H), 1.90 (m, 2H); cyclohexyl ethyl H: δ 1.10 (m, 11H), 2.00 (br, 2H), 2.20 (m, 2H).

Compound No. 7, yield: 75%; oil; MS: m/e 283 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.60 and 6.70 (s, 1H); piperidine H: complex, δ 4.60 (d, 2H), 3.90 (d, 2H), 3.10 (m, 3H), 1.80 (m, 2H); phenyl ethyl H: δ 7.30 (m, 5H,) 2.10 (br, 2H), 1.50 (m, 2H).

Compound No. 8, yield: 69%; M.P.: 151°C; MS: m/e 327 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.65 and 6.80 (s, 1H); piperidine H: complex, δ 4.70 (d, 2H), 4.50 (d, 2H), 3.60 (m, 1H), 2.80 (m, 2H), 2.10 (m, 2H); adamantyl acetyl H: δ 1.80 (m, 12H), 3.10 (m, 2H), 4.05 (m, 1H).

Compound No. 9, yield: 62%; M.P.: 148°C (decomposed); MS: m/e 357 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.60 and 6.85 (s, 1H); piperidine H: complex, δ 4.50 (d, 2H), 4.05 (m, 3H), 3.40 (d, 2H), 2.10 (m, 2H); dicyclohexyl acetyl H: δ 1.50 (m, 22H), 2.50 (m, 1H).

Compound No. 10, yield: 64%; oil; MS: m/e 281 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.75 and 6.60 (s, 1H); piperidine H: complex, δ 4.70 (d, 2H), 4.20 (m, 3H), 2.80 (m, 2H), 2.10 (d, 2H); phenyl vinyl H: δ 7.40 (m, 5H), 6.50 (m, 2H).

Compound No. 11, yield: 62%; oil; MS m/e 351 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.50 and 6.40 (s, 1H); piperidine H: complex, δ 4.60 (d, 2H) 4.10 (m, 3H), 2.80 (d, 2H), 1.80 (m, 2H); phenyl cyclohexyl acetyl H: δ 7.20 (m, 5H), 1.80 (m, 11H), 3.70 (m, 1H).

Compound No. 12, yield: 72%; M.P.: 136°C; MS:m/e 304 (M+); ^{1}H NMR ($CDCl_3$): imidazole H: δ 7.70 and 6.80 (s, 1H); piperidine H: complex, δ 4.60 (d, 2H), 4.00 (m, 2H), 3.60 (m, 3H), 1.88 (m, 2H); cyclohexyl propyl H; complex, δ 1.20 (m, 17H).

6.1.2. ALTERNATIVE METHOD FOR THE PREPARATION OF 4-(1-CYCLOHEXYLVALEROYL-4-PIPERIDYL) 1H-IMIDAZOLE (COMPOUND 1)

Preparation of acid anhydride: Triethylamine (1.01 g, 10.00 mmol) was slowly added to a stirred solution of 1.84 g (10.00 mmol) cyclohexylpentanoic acid in 60 ml acetonitrile at 0°C. After 30 min. of stirring, 1.08 g (10.00 mmol) of ethylchloroformate was added slowly in 5-7 min., so that the temperature remained between 0°C and 5°C. After 1h stirring, the solution was used for the preparation of Compound 1.

Preparation of Compound 1: The freshly prepared acid anhydride was poured into a suspension of 1.54 g (10.20 mmol) of 4(4-piperidyl)imidazole and 1.03 g (10.20 mmol) triethylamine in 70 ml acetonitrile. After 1 h of heating at 80°C, the solution was concentrated under reduced pressure, and the oily residue was taken up with 75 ml water and then extracted with 150 ml ethylacetate. The residual oil

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was obtained, which crystallized on addition of ethylacetate/hexane. Yield: 74%.

This method provides the desired amide in good yield when the piperidylimidazole is added in slight molar excess, e.g., about a 1.01 to 1 molar ratio, to the asymmetric anhydride.

Compounds No. 52-58 in Table I were synthesized in similar manner, i.e., by condensation of the asymmetric ethylchloroformate acid anhydride with 4(4-piperidyl) 1H-imidazole in the presence of triethyl amine.

Commercially available 3,3-diphenylpropionic acid and 4,4-diphenylbut-3-enoic acid were used as the starting materials for compounds 52 and 54, respectively. The unsaturated alkene bond of 4,4-diphenylbut-3-enoic acid was reduced under mild conditions by Pd/C (5%)/H_2 catalysis. This intermediate was then used to synthesize compound 53. Both intermediates 3,3-diclohexylpropionic acid and 4,4-dicyclohexylbutanoic acid, used in the preparation of compounds 55 and 56, respectively, were prepared by reduction of 3,3-diphenylpropionic acid and 4,4-diphenylbutanoic acid in the presence of catalyse Rh/alumina (5%)/H_2, 5 atm.

Compound No. 52, yield: 69%; MS.:M/e 359 (M+); ^1H NMR $CDCl_3$:imidazole H: δ 7.50 and 6.70 (s, 1H); piperidine H: complex δ 4.60 (d, 2H), 3.10 (m, 3H), 2.60 (d, 2H), 1.40 (m, 2H); propionyl H: complex δ 3.05 (m, 1H), 2.00 (d, 2H); biphenyl H: complex δ 7.20 (m, 10H), MA.: calc. C=76.85, H=7.00, N=11.68; found, 76.32, 6.72, 10.89, respectively.

Compound No. 53, yield: 73%; MS.:M/e 373 (M+); ^1H NMR $CDCl_3$:imidazole H: δ 7.65 and 6.70 (s, 1H); piperidine H: complex δ 4.60 (d, 2H), 3.00 (m, 2H), 2.50 (m, 2H), 1.80 (m, 2H); butanoyl H: δ 3.05 (m,

2H), 2.40 (m, 2H), 3.40 (m, 2H); diphenyl H: δ 7.10 (m, 10H).

Compound No. 54, yield: 64%; MS.:M/e 371 (M+); ^{1}H NMR $CDCl_3$:imidazole H:δ 7.40 and 6.50 (s, 1H); piperidyl H: complex δ 4.50 (d, 2H), 3.60 (m, 3H), 3.20 (d, 2H), 1.50 (d, 2H); butenyl H: complex δ 6.70 (d, 1H), 3.50 (d, 2H); diphenyl H:δ 7.10 (m, 10H).

Compound No. 55, yield: 75%; MS.: M/e 371 (M+); ^{1}H NMR $CDCl_3$:imidazole H:δ 8.00 and 7.10 (s, 1H); piperidyl H: complex δ 4.50 (d, 2H), 3.10 (d, 2H), 2.80 (m, 3H), 1.90 (d, 2H); propionyl H: complex δ 2.60 (d, 2H), 2.00 (m, 1H); dicyclohexyl H: complex δ 1.50 (m, 22H).

Compound No. 56, yield: 68%; MS.: M/e 385 (M+); ^{1}H NMR $CDCl_3$: imidazole H:δ 8.00 and 7.05 (s, 1H); piperidyl H: complex δ 4.50 (d, 2H), 3.80 (d, 2H), 3.00 (m, 3H), 2.10 (m, 2H); butanoyl H: δ complex 2.80 (m, 2H), 1.80 (m, 2H), 1.40 (m, 1H); dicyclohexyl H: complex δ 1.20 (m, 22H).

6.1.3. PREPARATION OF 4-(t-BUTOXY CARBONYL-4-PIPERIDYL) 1H-IMIDAZOLE (COMPOUND 2)

To a suspension of 224 mg (1.00 mmol) of 4-(4-piperidyl)-1H-imidazole dihydrochloride in 10 ml of methanol was added 202 mg (2.00 mmol) of triethylamine (the suspension turned to a clear solution) followed by dropwise addition of 218 mg (1.00 mmol) of di-t-butyl dicarbonate in 5 ml methanol over a period of 10 min. The reaction mixture was stirred at 25°C for 6 h, at the end of which the volatile materials were removed *in vacuo*. The oily residue was partitioned between 50 ml chloroform and 25 ml water. The organic layer was washed with 50 ml brine solution, then dried over anhydrous sodium sulfate. After filtration and removal of solvent, a pale yellow oil was obtained. The oil was treated with a mixture of methanol:

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petroleum ether (10:90). The resulting mixture was agitated vigorously with a glass rod until a solid appeared. After filtration and drying, the desired product was obtained as a white power. Yield: 65%; M.P.: 198°C; MS: m/e 251 (M^+); ^1H NMR ($CDCl_3$): imidazole H: δ 7.60 (s, 1H) and 6.60 (s, 1H); piperidine H: δ 4.20 (d, 2H), 2.80 (m, 4H), 2.20 (d, 2H), 1.60 (m, 1H), t-BOC H: 1.45 (s, 9H).

Compounds No. 13 and 14 in Table I were synthesized in similar manner. The pure product was obtained by preparative TCL Silica GEL GF, 60 (2000 microns), and the solvent of recrystallization was methanol:anhydrous ether (20:80).

Compound No. 13, yield: 78%; M.P.: 180°C; MS: m/e 255 (M+); ^1H NMR ($DMSOd_6$): imidazole H: δ 7.95 and 6.80 (s, 1H), NH: δ 7.80 and 6.60 (d, 1H); piperidine H: complex, δ 4.50 (d, 2H), 3.60 (m, 3H), 3.10 (m, 1H), 2.75 (m, 2H); phenyl H: δ 7.40 (m, 5H); MA: (C,H,N,): 70.36%, 6.71%, 16.30%.

Compound No. 14, yield: 72%; M.P.: 185°C; ^1H NMR ($CDCl_3$); imidazole H: δ 7.60 and 6.80 (s, 1H); piperidine H: complex, δ 4.50 (d, 2H), 3.00 (m, 3H), 2.05 (d, 2H), 1.60 (m, 2H); t-butyl H: δ 1.10 (s, 9H).

6.1.4. PREPARATION OF 4(-4-PIPERIDYL)-1H-IMIDAZOLE

In a preferred embodiment, 4(4-piperidyl)-1H-imidazole for use in the synthesis of the H_3-receptors antagonists is prepared according to the following method.

Bromination of 4-acetyl piperidine (Aldrich) in hydrogen bromide/acetic acid was performed as described (Barlin et al., *Aust. J. Chem* 42:735 (1989)).

A mixture of 11.23g (4.00 mmol) of bromoacetyl pyridine and 3.98 ml (10.0 mmol) formamide were fused together at 110°C with stirring for 4h. The crude reaction mixture was then concentrated on the rotary evaporator to remove volatile matter. The residue was dissolved in 50 ml methanol, and to this solution was added 100 ml anhydrous dimethyl ether slowly with stirring, which led to the formation of a brown precipitate. After stirring for another 0.5h, the precipitate was filtered, washed with 50 ml anhydrous ether and dried. This solid residue was dissolved in 20 ml water and the aqueous solution was basified to pH 9 with sodium carbonate. To this solution was added 150 ml absolute ethanol slowly with stirring till a solid formed, which was filtered off. The filtrate was heated to boiling, then treated with activated carbon and filtered. The filtrate was concentrated on rotary evaporator to dryness. Yield: 3.36g 58%; M.P.: 152°C (decomposed); MS: m/e 145 (M+), ^{1}H NMR (D_2O): imidazole H: δ 7.80 (s, 1H) and 7.20 (s, 1H); pyridyl H: 8.10 (d, 2H), 7.17 (d, 2H). The pyridyl moiety was reduced by catalytic hydrogenation using 5-10% rhodium on carbon in acidified water at 20-55 atmospheres as described (Schunack, *Archiv. Pharma*. 306:934 (1973)).

6.2. ANTAGONIST ACTIVITY IN VITRO

The various compounds were tested for the ability to bind to the histamine H_3 receptor. A binding assay in a rat brain membrane preparation, based on inhibition of binding of [^{3}H]-N-alpha-methylhistamine using excess unlabeled alpha-methylhistamine to account for nonspecific binding, was developed. Total, specific and nonspecific binding of [^{3}H]-N-alpha-methylhistamine to brain membranes is shown in

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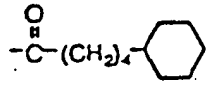
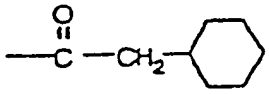
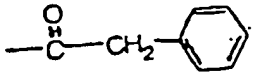
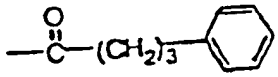
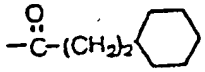
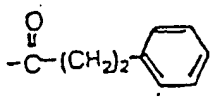
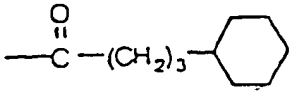
FIG. 1. The K_d value was 0.19 nM in this preparation and the nonspecific binding was less than 20% of the total binding at the Kd value. The compounds thioperamide (Arrang et al., *Nature* 327:117-123 (1987)) and burimamide (Black et al., *Nature* 236:385-390 (1972)) were tested as controls for this assay. The results are shown in Table I.

TABLE I

4-Piperidyl (imidazole) Compounds and Their Activities on Rat Brain Membranes.

(^{3}H-N$^\alpha$-methylhistamine as Radioligand)

Cmpd No.	R_1	X (= CO-(O)$_t$-(CH$_2$)$_r$-R)	IC_{50} (nm)	M.P.
Thioperamide	H		4.0±0.6 n=4	170°C
		Burimamide	156±57	
1	H		23±6 n=3	159°
3	H		19±12 n=3	Oil
4	H		1400±437 n=3	Oil
5	H		262±9 N=3	Oil
6	H		34±1.4 n=3	Oil
7	H		34.1±3.6 n=3	Oil
12	H		41.4±9 n=3	136°C

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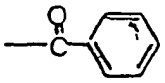
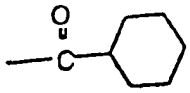
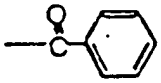
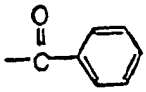
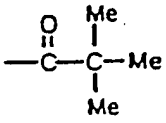
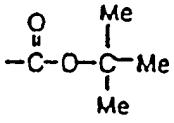
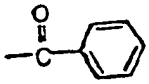
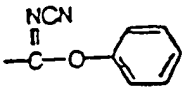
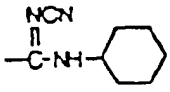
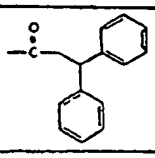
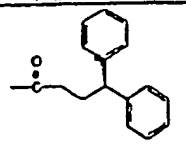
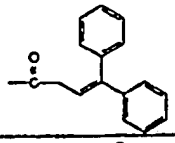
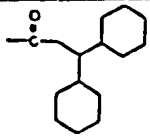
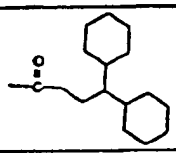
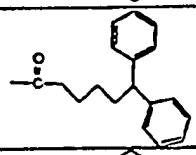
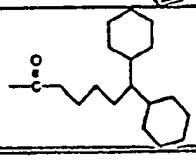
Cmpd No.	R_1	X (= $CO-(O)_t-(CH_2)_n-R$)	IC_{50} (nm)	M.P.
13	H		151±44 n=4	180°C
41	H		inactive n=2(1μM)	192°C
42	CH_3		inactive n=2(1μM)	Oil
43	X		inactive n=3	99°C
44	X		inactive n=2	81°C
45	X		inactive n=2	79°C
46	$PhCH_2$		inactive n=2	62°C
47	H		231 n=1	185°C
48	H		inactive n=2	168°C
52	H		93.1	129-131°C

Cmpd No.	R_1	X (= CO-(G)$_z$-(CH$_2$)$_n$-R)	IC_{50} (nm)	M.P.
53	H		124	oil
54	H		1000	158°C decomp.
55	H			118°C decomp.
56	H			152°C decomp.
57	H			
58	H			

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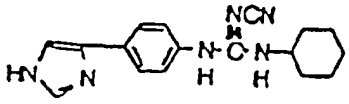
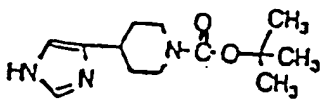
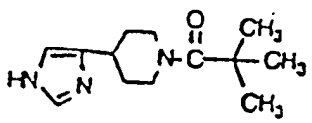
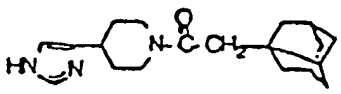
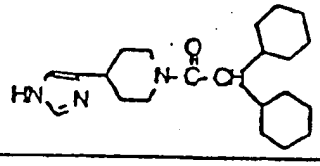
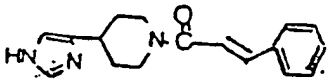
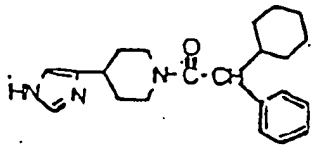
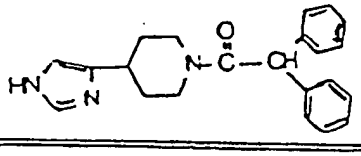
Cmpd. No.	Structure	IC_{50}(Nm)	M.P.
50		inactive n=2(μM)	148.5°-150.5°C
2		243.5±1.9 n=2	198°C
14		inactive n=2	185°C
8		inactive n=2	151°C
9		inactive n=2	148°C
10		570±172 n=3	Oil
11		260±38 n=2	Oil
51			115°C

6.3. DISCUSSION

The results in Table I show that the compounds of the invention are effective for binding to the histamine H_3-receptor. Interestingly, cyanoguanidine derivatives (e.g., compounds 47, 48 and 50) were ineffective at binding to the H_3-receptor. This result is in contrast to earlier observations about H_2-receptor antagonists. With H_2-receptor antagonists, cyanoguanidine and thiourea-containing derivatives (cimetidine and metiamide, respectively) were found to be bioisosteres, i.e., functionally substantially equivalent (Brimblecombe et al., *Gastroenterology* 74: 339-347 (1978)).

7. PHARMACOLOGICAL EVALUATION IN THE CNS

A representative compound, 1, was tested *in vivo* for (1) the ability to penetrate the blood brain barrier; and (2) the effect of behavior in mice.

7.1. PENETRATION OF THE BLOOD-BRAIN BARRIER

Blood-brain barrier penetration in rats was assessed by an *ex vivo* binding procedure. Young adult male Long-Evans rats were injected i.p. with saline or H_3 antagonists in saline. At various times after injection animals were sacrificed, the cortex was removed, homogenized in 50 mM Na/K-phosphate buffer, pH 7.4, and the binding of 1 nM [^{3}H]-N$^\alpha$-methylhistamine was measured using 400 µg protein of the homogenate. Nonspecific binding was accounted for by the inclusion of excess thioperamide in some samples. Under these conditions, the binding was approximately 90% specific.

As shown in FIG. 2, thioperamide at doses of 2, 5, and 10 mg/kg, when measured 15 min after injection, decreased the binding of [^{3}H]-N$^\alpha$-methylhistamine to H_3

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receptors in the cortex. This means that the thioperamide at these doses and after this time was able to penetrate the blood-brain barrier. Figure 3 shows that compound 1 also penetrates the blood-brain barrier one hour after injections of doses of 50 and 70 mg/kg. Taking into account the difference in affinity comparing thioperamide (4.0 nM) and compound 1 (23 nM), these data suggest that compound 1 penetrates the blood-brain barrier at least as well as thioperamide.

7.2. BEHAVIORAL EFFECTS IN MICE

The overall strategy to show central nervous system antagonist activity was to challenge effects of the agonist (R)α-methylhistamine. Therefore, the first objective was to establish a dose response curve for behavioral effects of (R)α-methylhistamine. Male albino CF-1 mice weighing 20-30 g were used. Saline or (R)α-methylhistamine in saline was injected i.p. in a volume ≤ 0.4 ml. Animals were observed for various behaviors three times for 10 seconds during each 10 minute interval for a total of 2 hours. Animals were scored for the presence (1) or absence (0) of the behavior and the results were reported as the accumulated score for a 30 minute period (maximum score = 9). As shown in FIG. 4, (R)α-methylhistamine produced a dose-dependent (range of 15 to 35 mg/kg) increase in sleeping one hour after injection. The effect was also evident at 30 minutes after injection.

To assess the effects of antagonists, they were administered with the (R)α-methylhistamine in saline. FIG. 5 shows that thioperamide was able to inhibit the soporific effect of 30 mg/kg of (R)α-methylhistamine. With thioperamide alone (i.e., in the absence of the α-methylhistamine H_3 receptor agonist), animals were

very active, exhibiting normal behaviors. FIG. 6 shows that compound 1 inhibited the soporific effect of 25 mg/kg (R)α-methylhistamine.

7.3. DISCUSSION

The results of the *in vitro* (see section 6, *supra*) and *in vivo* activity assays show that a compound of the invention is useful for increasing histamine activity in the brain.

In the foregoing *in vivo* assays, thioperamide was used as a positive control. The results indicate that compound 1 is effective as an H_3-receptor antagonist. Direct comparison of the two compounds is not available from the data, however, since the experimental protocols used to test each were not identical.

It is noteworthy that in all testing to date, no toxicity of the 1 compound has been observed, even at high doses.

8. SPECIFICITY OF COMPOUND 1

The selectivity of action of compound 1 for histamine H_3-receptors was determined in a NOVASCREEN™ receptor selectivity study. At concentrations of 10^{-5} M, no significant binding to adenosine, excitory or inhibitory amino acid, dopamine, serotonin, or a broad range of petidergic receptors, or to ion channel proteins, peptide factor or second messenger systems was observed. The binding study results are shown in Table II.

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TABLE II

NOVASCREEN™ RECEPTOR SELECTIVITY ASSAY

Receptor/ Selectivity	Reference Compound	Reference K_i(nM)	Initial Percent Inhibition (Average; N=2) 10^{-5}M
Adenosine			
Adenosine	NECA	120.00	-3.0
Amino Acids			
Ecitatory			
Quisqualate	Quisqualic Acid	11.80	-1.8
Kainate	Kainic Acid DME	24.93	42.1
MK-801	MK801	4.30	-8.6
NMDA	NMDA	359.00	-4.5
PCP	PCP	62.30	9.7
Glycine	Glycine	300.00	1.8
Inhibitory			
Glycine	Strychinine Nitrate	33.50	17.4
$GABA_A$	GABA	2.80	0.6
$GABA_B$	GABA	176.00	0.0
Benzodiazephine	Clonazepam	3.40	2.7
Biogenic Amines			
Dopamine 1	Butaclamol	37.30	6.4
Dopamine 2	Spiperone	0.08	3.5
Serotonin 1	Serotonin	4.60	-3.6
Serotonin 2	Serotonin	531.00	10.5
Peptides			
Angiotensin	Angiotensin II	0.20	6.5
Arg-Vasopressin V_1	arg-Vasopressin	4.90	10.1
Bombesin	Tyr4-Bombesin	0.55	-5.5
CCK Central	CCK	0.13	18.6
CCK Peripheral	CCK	0.02	6.9
Substance K	Neurokinin A	2.75	29.2
Substance P	Substance P	0.08	20.0
NPY	Neuropeptide Y	0.50	-8.7
Neurotensin	Neurotensin	1.23	-10.5
Somatostatin	Somatostatin	0.03	4.1
VIP	VIP	1.53	17.1

Receptor/ Selectivity	Reference Compound	Reference K_i(nM)	Initial Percent Inhibition (Average; N=2) 10^{-5}M
Channel Proteins			
Calcium	w-Conotoxin	0.01	1.9
Calcium	Nifedipine	1.60	8.1
Chloride	TBPS	112.40	-3.4
Potassium	Apamin	0.05	7.7
Peptide Factors			
ANF (rat)	ANP	0.15	0.1
EGF	EGF	0.24	18.1
NGF	NGF	0.80	17.1
Second Messenger Systems			
Adenylate Cyclase Forskolin	Forskolin	29.40	2.1
Protein Kinase C			
Phorbol Ester	PDBU	16.50	0.9
Inositol Triphosphate	IP3	12.50	9.2

Values are expressed as the percent inhibition of specific binding and represent the average of duplicate tubes at each of the concentrations tested. Bolded values represent inhibition of fifty percent or greater.

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9. DEMONSTRATION OF APPETITE SUPPRESSION ACTIVITY IN VIVO

Male Sprague-Dawley rats (200-300 grams) were used in all experiments. Rats were fasted for 24 hours prior to all experiments. Water was provided ad lib. Rats were singly placed in a ventilated plastic circular rat metabolism chamber with a stainless steel wire mess grid flood equipped with food and H_2O attachments. A measured quantity of food (50 grams) and water (100 mls) was supplied through attachments to the metabolism chambers. Food and water intake was measured at the 2, 4 and 6 hour time periods.

Immediately prior to placement of the rats in the metabolism chambers, animals were administered interperitoneally, vehicle or compound 1 (3, 10 or 30 mg/kg). Final volumes were 1.0 ml/kg. Subsequently, they were left undisturbed for the experimental period except when the food and H_2O attachments were briefly removed for measurement of food and water intake. At the completion of the experiment, animals were returned to their home cages. The results are found in Table III and Figures 7 and 8.

Table III

The Effect of Food Intake In Rats Following The Administration of Compound I

	Column 1	2 HR	S.E.	4 HR	S.E.	6 HR	S.E.
1	VEHICLE	6.167 ±	0.366	7.083 ±	0.358	13.000	
2	3 mg/kg	5.687 ±	0.494	7.333 ±	0.558		
3	10 mg/kg	2.000 ±	0.258	5.167 ±	0.601		
4	30 mg/kg	1.167 ±	0.477	2.333 ±	0.615	5.000 ±	0.940

The above protocol is repeated for the histamine H_3 receptor antagonists disclosed herein.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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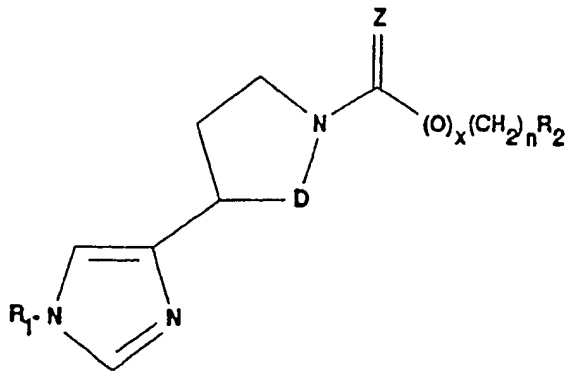
WHAT IS CLAIMED IS:

1. Use of a compound of the formula:

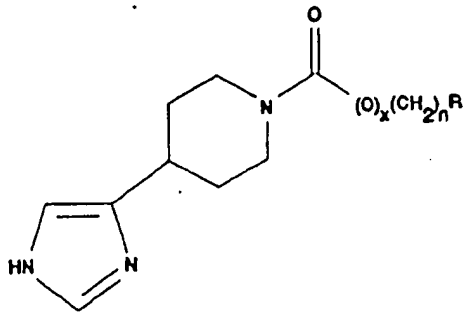


I

wherein R_1 represents hydrogen, an *in vivo* hydrolyzable group, an alkyl group, an cyclic alkyl group, or an aryl group; D is CH_2 or CH_2CH_2; Z is S or O; x is 0 or 1; n is an integer from 0 to 6; and R_2 represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 atoms, or a substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R_2 is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R_2 is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for suppressing appetite in a subject.

2. The use according to claim 1 in which said compound is a compound of the formula:



II

wherein x is 0 or 1; n is an integer from 0 to 6; and R represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 carbon atoms, or substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof.

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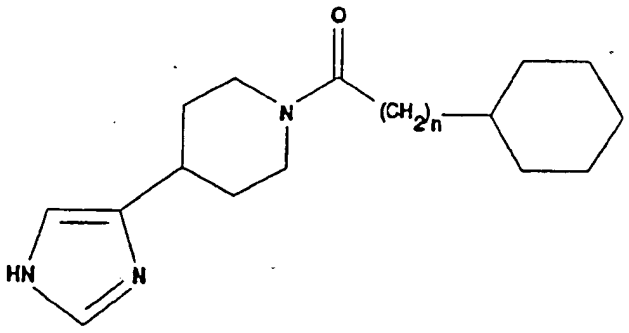
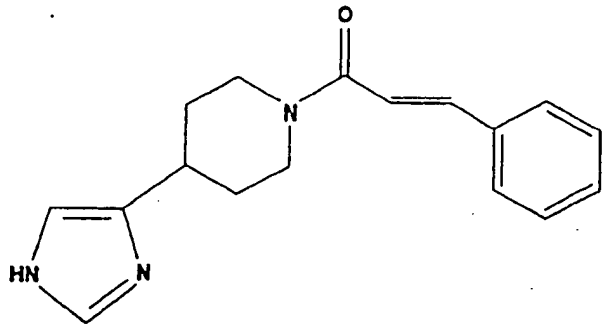
3. The use according to claim 2 in which said compound is a compound of the formula:



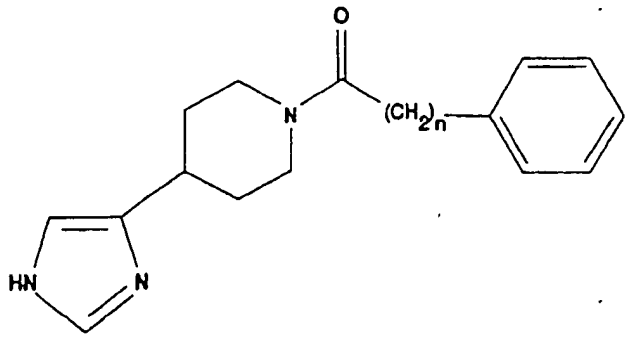
in which n is 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

4. The use according to claim 2 in which said compound is a compound of the formula:



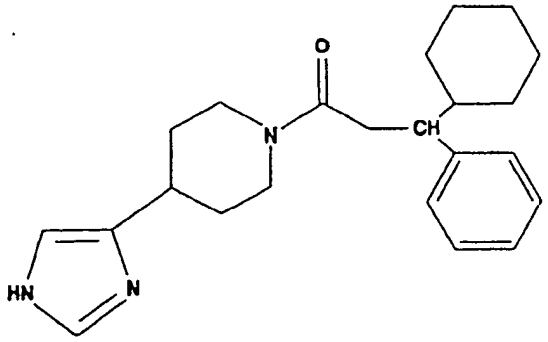
or a pharmaceutically acceptable salt thereof.

5. The use according to claim 2 in which said compound is a compound of the formula:



wherein n is 0, 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

6. The use according to claim 2 in which said compound is a compound of the formula:



or a pharmaceutically acceptable salt thereof.

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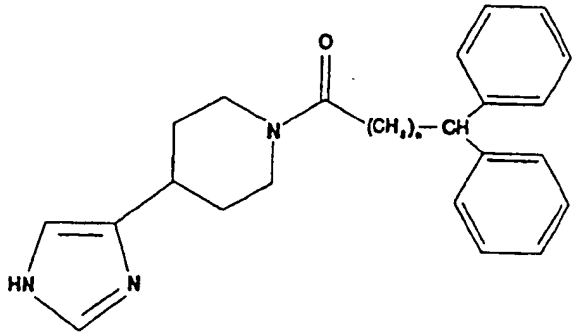
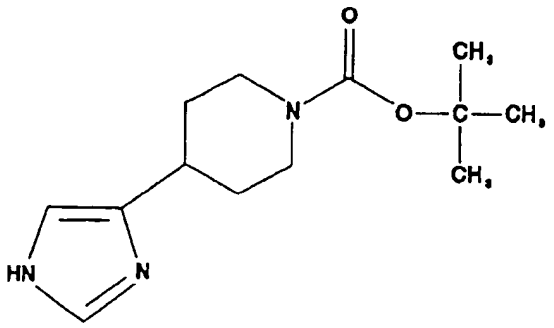
7. The use according to claim 2 in which said compound is a compound of the formula:



in which n is 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

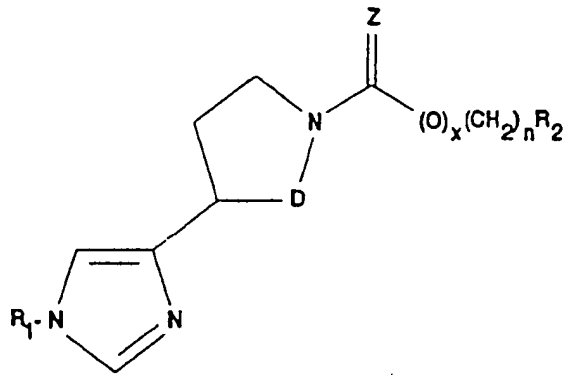
8. The use according to claim 2 in which said compound is a compound of the formula:



or a pharmaceutically acceptable salt thereof.

9. The use of compound 4-(1-cyclohexylvaleroyl-4-piperidyl)1H-imidazole or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for suppressing appetite in a subject.

10. A pharmaceutical composition comprising an amount of a compound of the formula:



I

wherein R_1 represents hydrogen, an *in vivo* hydrolyzable group, an alkyl group, an cyclic alkyl group, or an aryl group; D is CH_2 or CH_2CH_2; Z is S or O; x is 0 or 1; n is an integer from 0 to 6; and R_2 represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 atoms, or a substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R_2 is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R_2 is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof, effective to achieve appetite suppression, and a pharmaceutically acceptable carrier or excipient.

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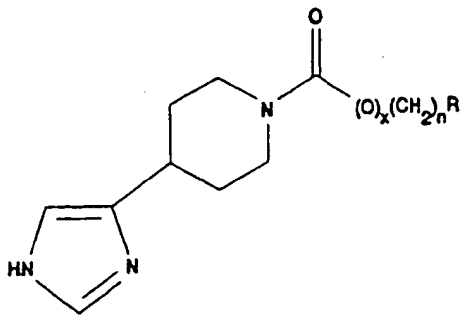
11. The pharmaceutical composition according to claim 10 wherein said compound is a compound of the formula:

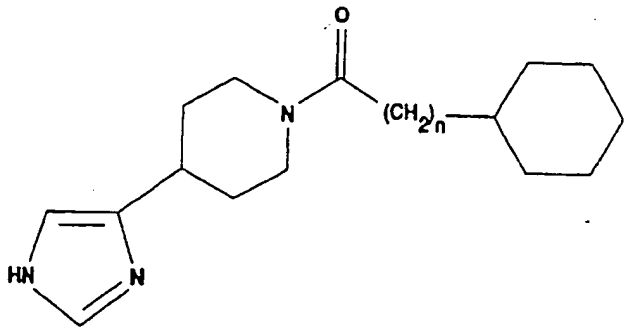


II

wherein x is 0 or 1; n is an integer from 0 to 6; and R represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 carbon atoms, or substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof.

12. The pharmaceutical composition according to claim 11 wherein said compound is a compound of the formula:



in which n is 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

13. A pharmaceutical composition comprising an amount of 4-(1-cyclohexylvaleroyl-4-piperidyl)1H-imidazole or a pharmaceutically acceptable salt thereof, effective to achieve appetite suppression, and a pharmaceutically acceptable carrier or excipient.

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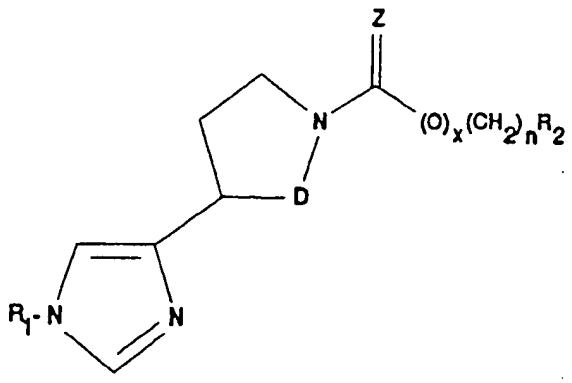
14. A method of suppressing appetite in a subject comprising administering to a subject in whom appetite suppression is desired, an effective amount of a compound of the formula:

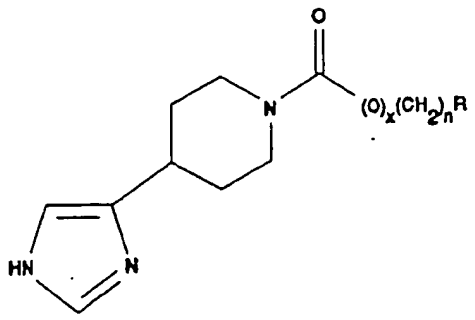


I

wherein R_1 represents hydrogen, an *in vivo* hydrolyzable group, an alkyl group, an cyclic alkyl group, or an aryl group; D is CH_2 or CH_2CH_2; Z is S or O; x is 0 or 1; n is an integer from 0 to 6; and R_2 represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 atoms, or a substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R_2 is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R_2 is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof.

15. The method according to claim 14 in which the compound is a compound of the formula:



II

wherein x is 0 or 1; n is an integer from 0 to 6; and R represents a substituted or unsubstituted linear chain or branched chain alkyl group of up to about 20 carbon atoms, a substituted or unsubstituted carbocyclic group of up to about 20 carbon atoms, or substituted or unsubstituted aryl group of up to about 20 carbon atoms, with the provisos that if R is *tert*-butyl, cyclohexyl, or dicyclohexylmethyl, x or n must not be 0; and if R is adamantane, the sum of x and n must be greater than 1;

or a pharmaceutically acceptable salt thereof.

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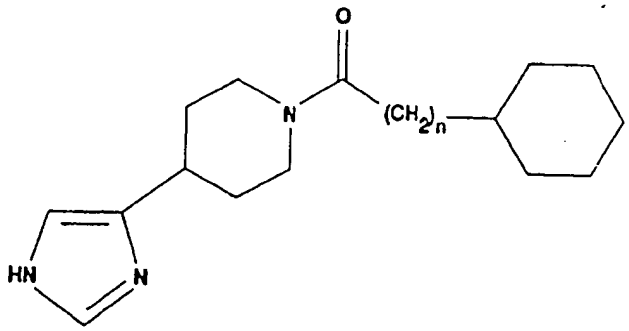
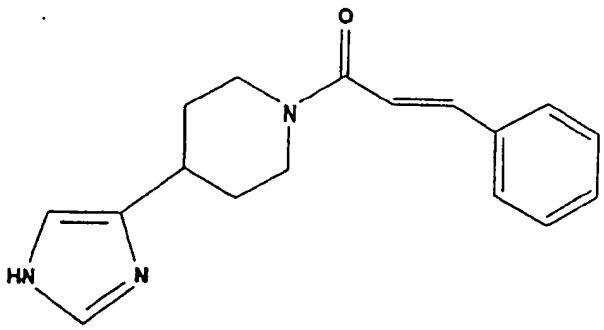
16. The method according to claim 15 in which the compound is a compound of the formula:



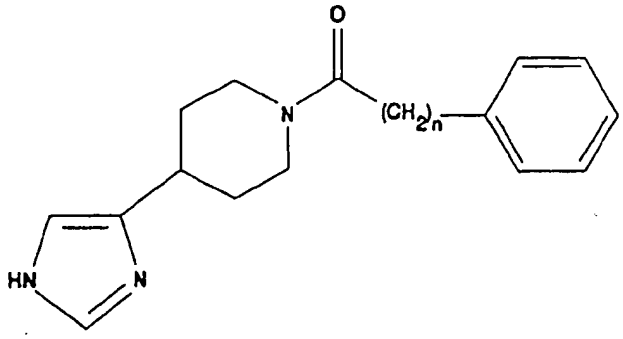
in which n is 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

17. The method according to claim 15 in which the compound is a compound of the formula:



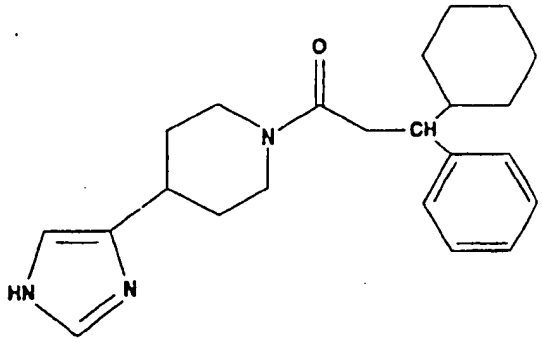
or a pharmaceutically acceptable salt thereof.

18. The method according to claim 15 in which the compound is a compound of the formula:



wherein n is 0, 1, 2, 3 or 4; or a pharmaceutically salt thereof.

19. The method according to claim 15 in which the compound is a compound of the formula:



or a pharmaceutically acceptable salt thereof.

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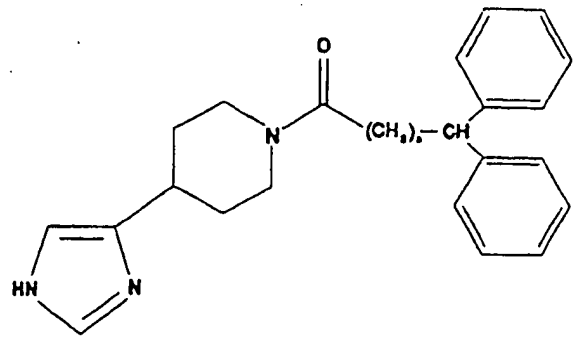
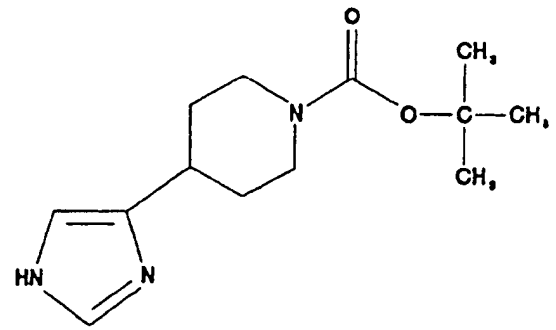
20. The method according to claim 15 in which the compound is a compound of the formula:



in which n is 1, 2, 3 or 4; or a pharmaceutically acceptable salt thereof.

21. The method according to claim 15 in which the compound is a compound of the formula:



or a pharmaceutically acceptable salt thereof.

22. The method according to any one of claims 14 to 21 in which the subject is human, and said administration is to treat obesity or weight gain.

23. The method according to claim 22 wherein said compound is administered orally.

24. The method according to claim 22 wherein said compound is administered parenterally.

25. The method according to claim 22 wherein the amount of said compound administered is about 0.01 mg/kg to about 200 mg/kg in a single dose or divided dose per day.

26. A method of suppressing appetite in a subject comprising administering to a subject in whom appetite suppression is desired, an effective amount of 4-(1-cyclohexylvaleroyl-4-piperidyl)1H-imidazole or a pharmaceutically acceptable salt thereof.

27. The method according to claim 26 in which the subject is human, and said administration is to treat obesity or weight gain.

28. The method according to claim 27 wherein said compound is administered orally.

29. The method according to claim 27 wherein said compound is administered parenterally.

30. The method according to claim 27 wherein the amount of said compound administered is about 0.01 mg/kg to about 200 mg/kg in a single dose or divided dose per day.

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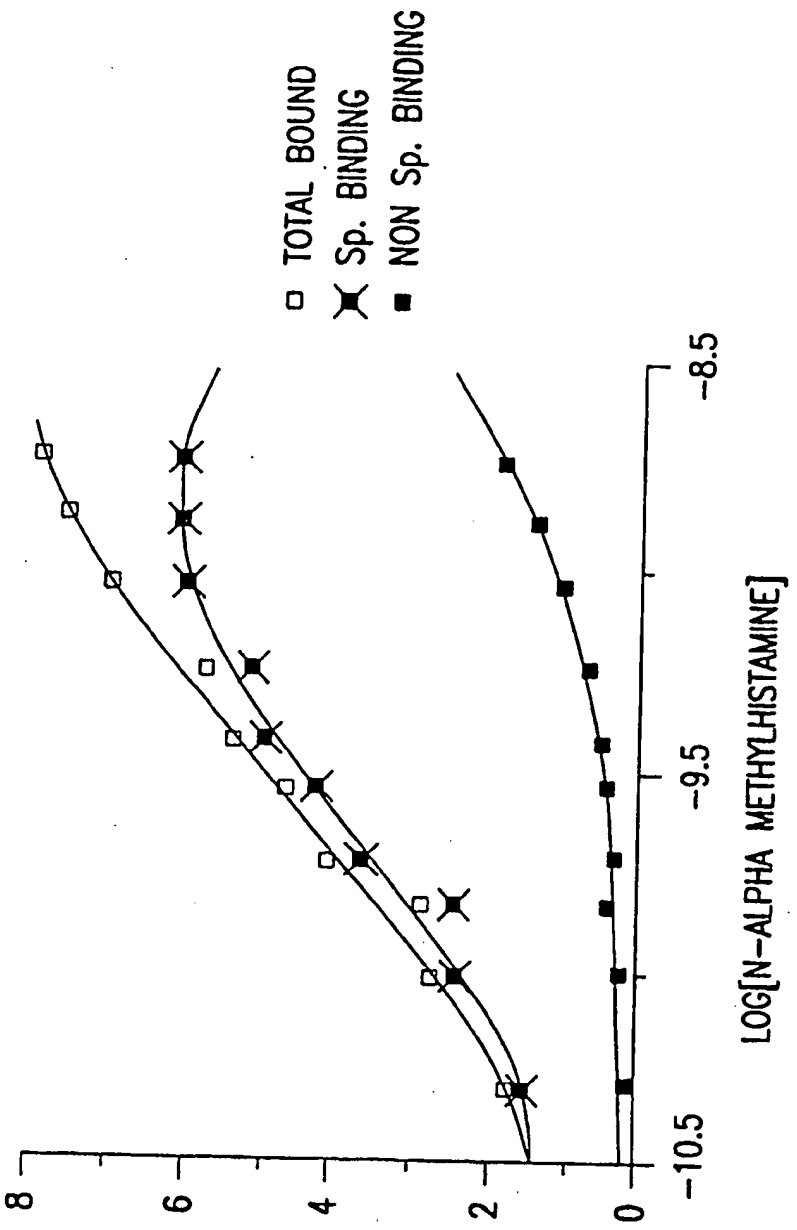
TOTAL BOUND
Sp. BINDING
NON Sp. BINDING
8
6
4
2
0
-10.5
-9.5
-8.5
LOG[N-ALPHA METHYLHISTAMINE]

FIG. 1

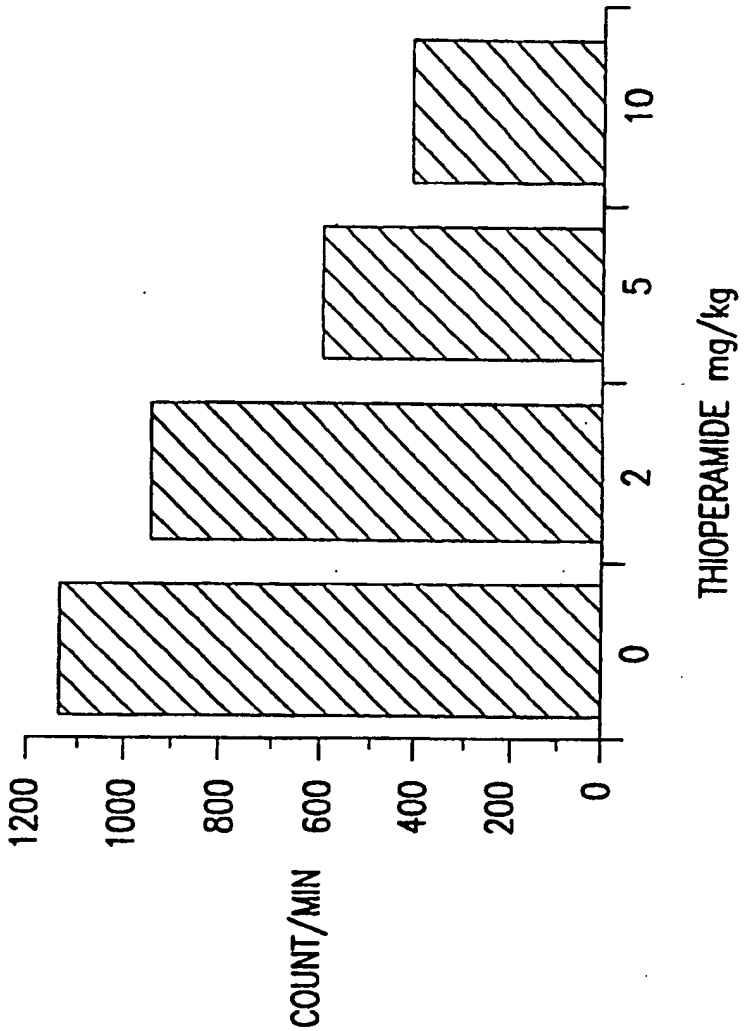

FIG.2

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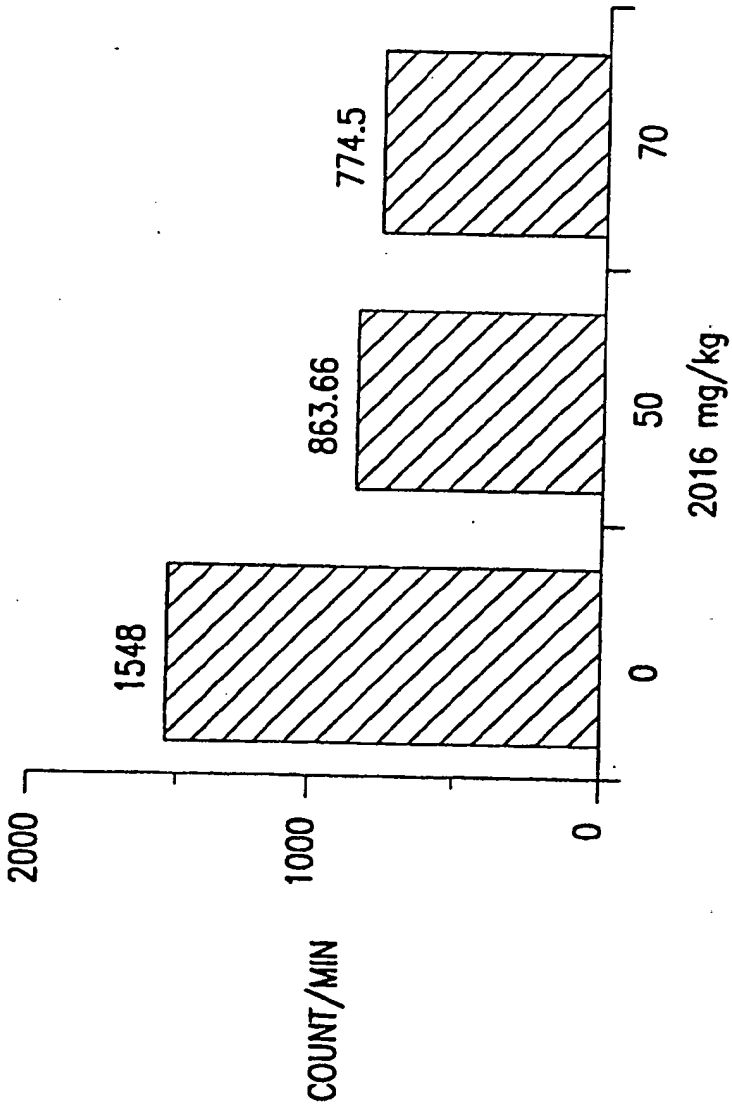

FIG.3

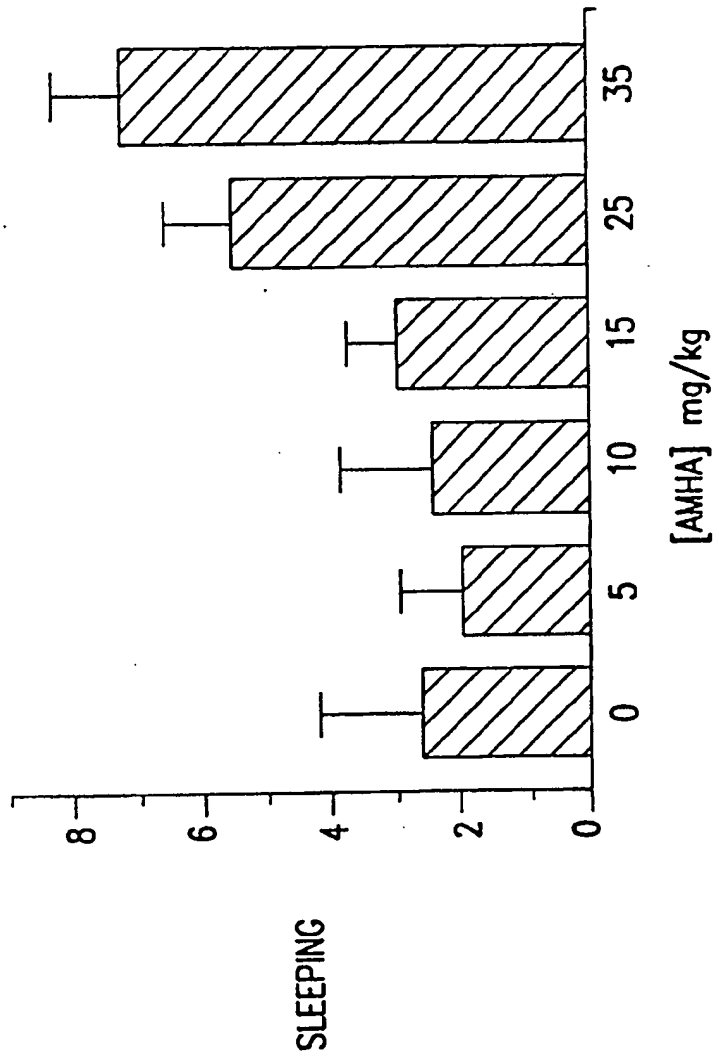

FIG.4

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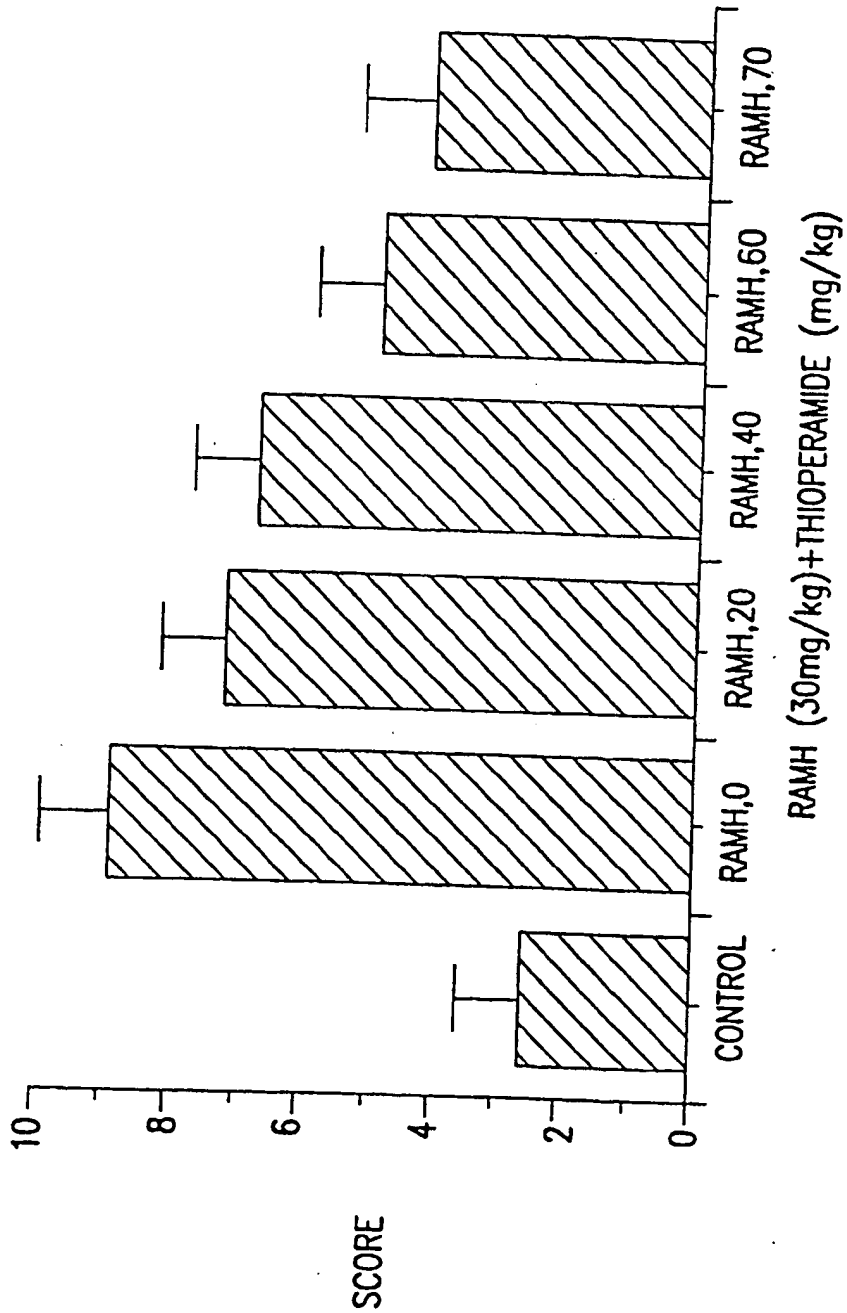
SCORE
10
8
6
4
2
0
CONTROL
RAMH,0
RAMH,20
RAMH,40
RAMH,60
RAMH,70
RAMH (30mg/kg)+THIOPERAMIDE (mg/kg)

FIG.5

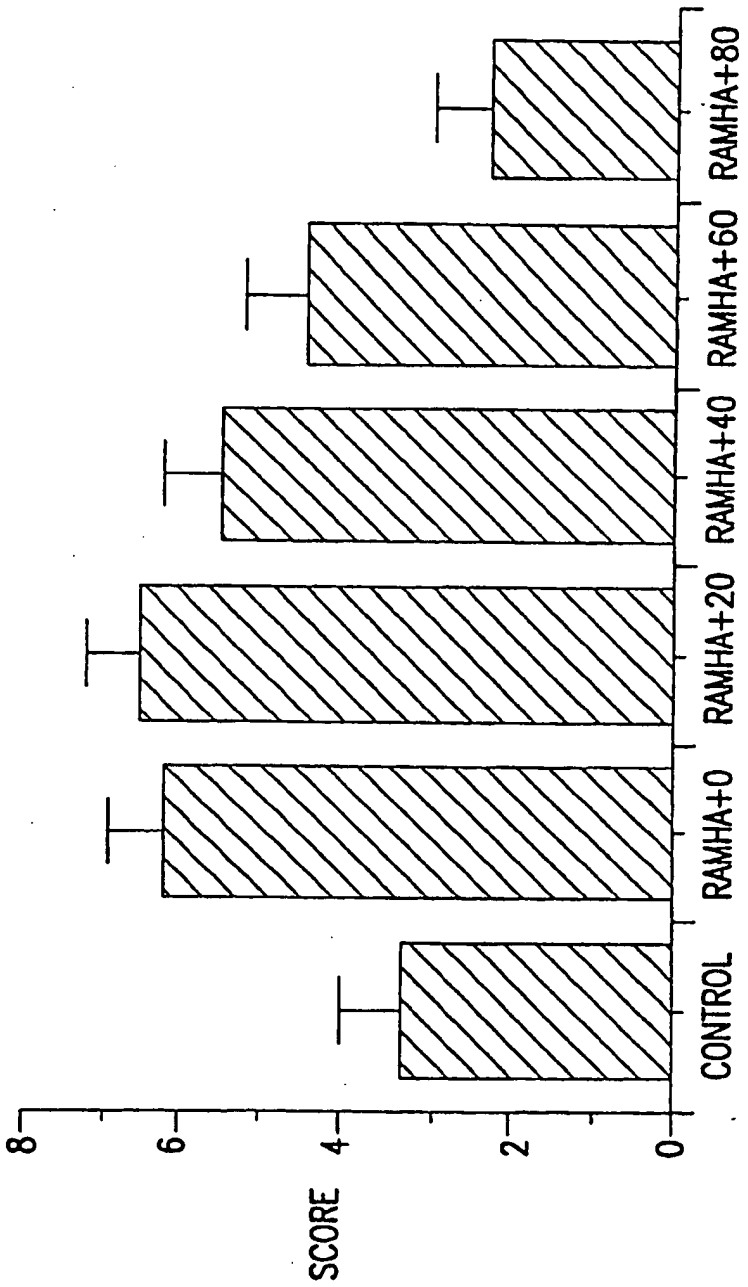

FIG.6

BNSDOCID: <WO___9511894A1_I_>

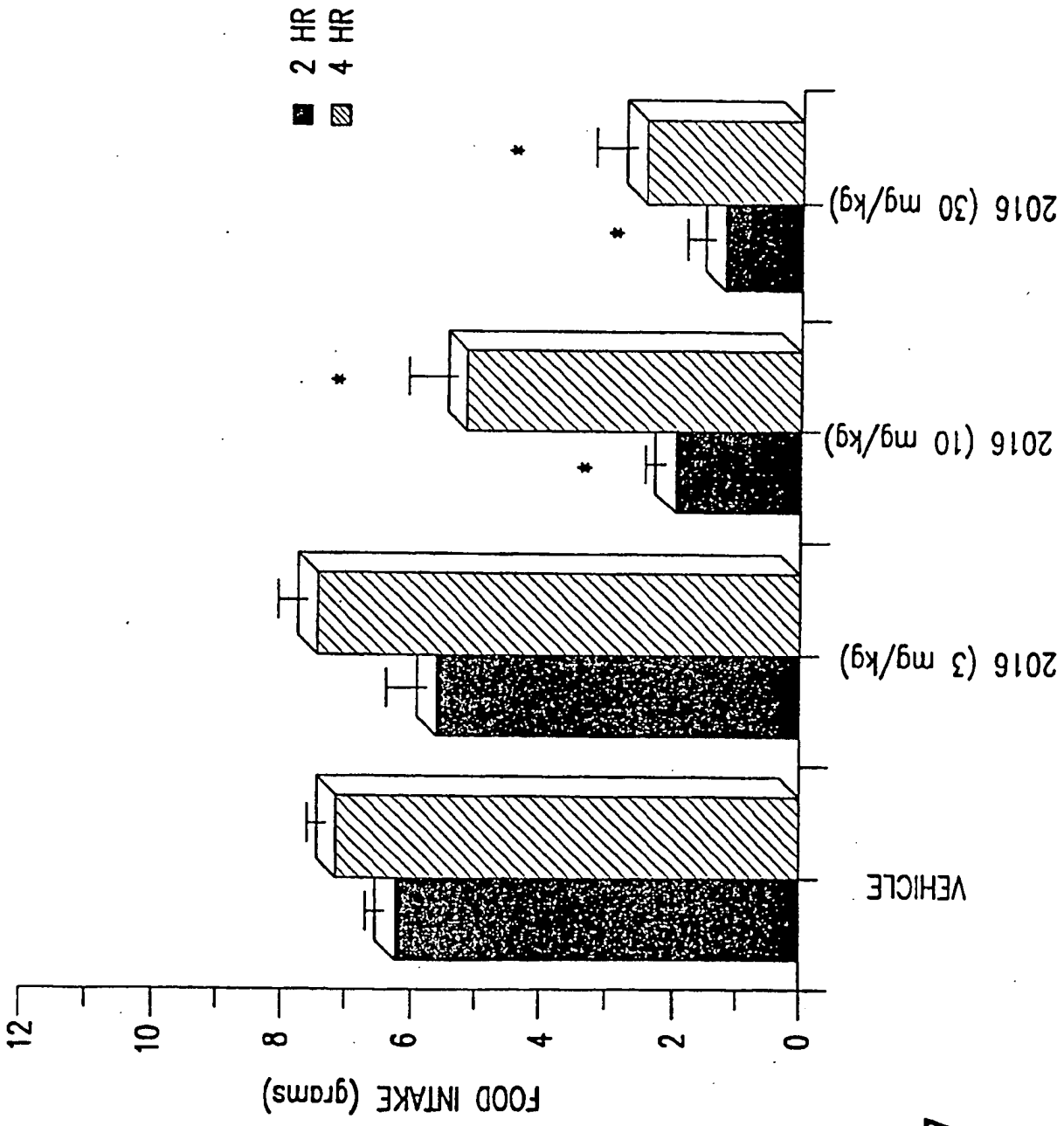
2 HR
4 HR
FOOD INTAKE (grams)
0
2
4
6
8
10
12
VEHICLE
2016 (3 mg/kg)
2016 (10 mg/kg)
2016 (30 mg/kg)
*
*
*
*

FIG.7

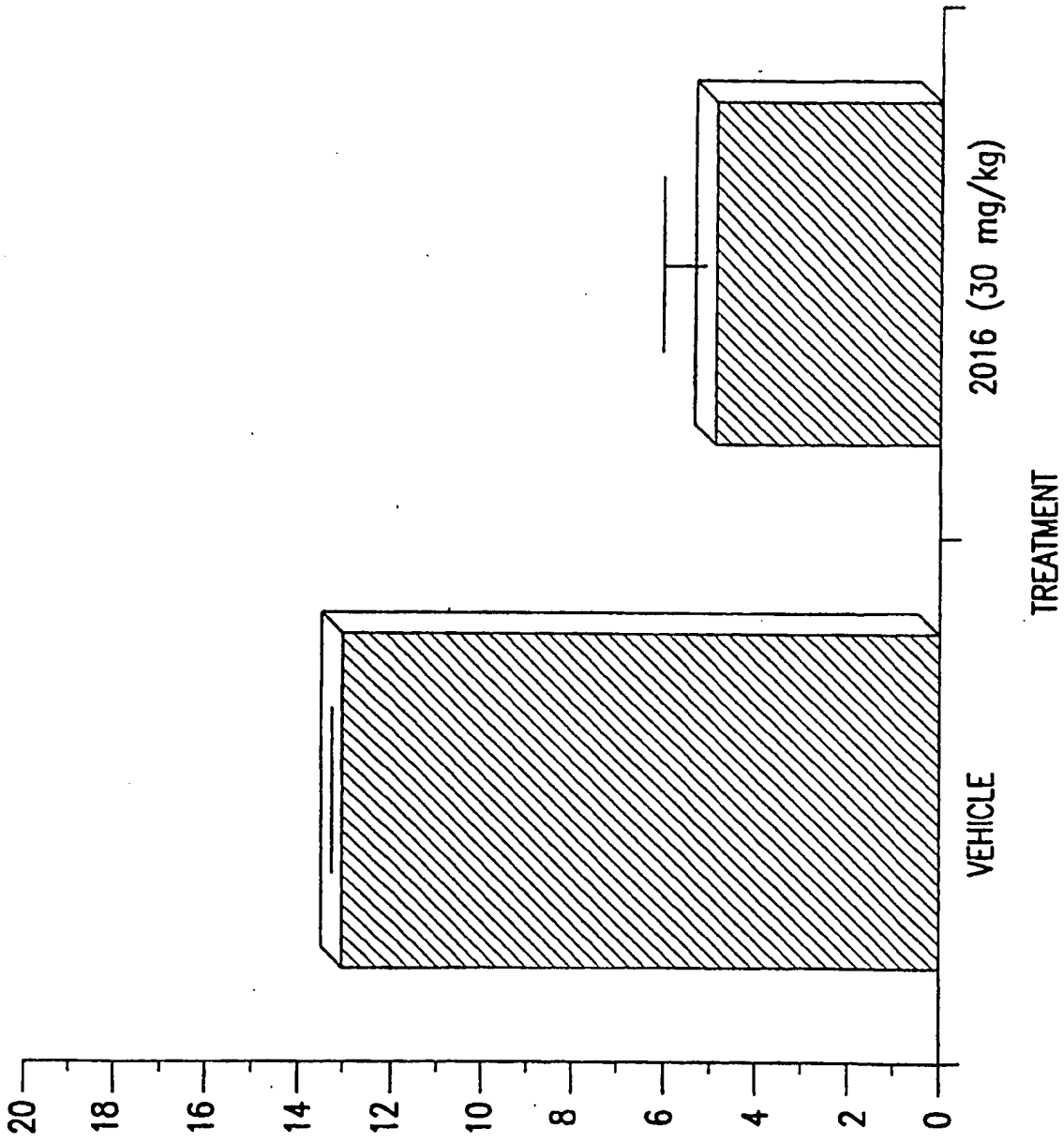

FIG.8

BNSDOCID: <WO___9511894A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11790

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07D 401/04; A61K 31/445
US CL :546/203, 210; 548/314.7; 514/319, 326, 397, 910
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 546/203, 210; 548/314.7; 514/319, 326, 397, 910

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A, 0,197,840 (ARRANG ET AL.) 15 OCTOBER 1986, see entire document.	1-46
A	US, A, 5,051,424 (WIERINGA) 24 SEPTEMBER 1991, see entire document.	1-46
A	US, A, 4,220,653 (VIVINO) 02 SEPTEMBER 1980, see entire document.	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
28 NOVEMBER 1994

Date of mailing of the international search report
13 JAN 1995

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Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
MICHAEL B. HYDORN
Telephone No. (703) 305-1235

Psychopharmacology (1999) 142:215–220

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ORIGINAL INVESTIGATION

Carmen Pérez-García · Lydia Morales
M. Victoria Cano · Isabel Sancho
Luis F. Alguacil

XP-000980226

Effects of histamine H_3 receptor ligands in experimental models of anxiety and depression

P.D. 1999
p. 215-220 = 6

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Abstract Histamine H_3 receptor ligands have been proposed to be of potential therapeutic interest for the treatment of different central nervous system disorders; however, the psychopharmacological properties of these drugs have not been studied extensively. In this work, we investigated the possible involvement of histamine H_3 receptor function in experimental models of anxiety (elevated plus-maze) and depression (forced swimming test). Male Sprague-Dawley rats were treated IP with the histamine H_3 receptor agonist *R*-α-methylhistamine (10 mg/kg) or the histamine H_3 receptor antagonist thioperamide (0.2, 2 and 10 mg/kg) and 30 min afterwards the time spent in the open arms of an elevated plus-maze was registered for 5 min. The immobility time of male OF1 mice in the forced swimming test was recorded for 6 min, 1 h after the IP administration of *R*-α-methylhistamine (10 and 20 mg/kg), thioperamide (0.2, 2, 10 and 20 mg/kg) or another histamine H_3 receptor antagonist, clobenpropit (5 mg/kg). The locomotor activity of mice was checked in parallel by means of an activity meter. Both saline controls and active drug controls were used in all the paradigms. Neither thioperamide nor *R*-α-methylhistamine significantly changed animal behaviour in the elevated plus-maze. *R*-α-methylhistamine and the higher dose of thioperamide assayed (20 mg/kg) were also inactive in the forced swimming test. By contrast, thioperamide (0.2–10 mg/kg) dose-dependently decreased immobility, the effect being significant at 10 mg/kg (33% reduction of immobility); clobenpropit produced an effect qualitatively similar (24% reduction of immobility). None of these histamine H_3 receptor antagonists affected locomotor activity. These preliminary results suggest that the histamine H_3 receptor blockade could be devoid of anxiolytic potential but have antidepressant effects. Besides, the stimulation of these receptors does not seem to be followed by changes in the behavioural parameters studied.

Key words Histamine H_3 receptor · *R*-α-methylhistamine · Thioperamide · Clobenpropit · Anxiety · Depression

Introduction

The role of histamine as a cellular mediator has been known for a long time, whereas the knowledge of its role as a neurotransmitter is more recent (Schwartz et al. 1991). In addition to the classical histamine receptors termed H_1 and H_2, Arrang et al. (1983) proposed a third receptor subtype that was initially described as a presynaptically located autoreceptor. Thus, activation of the histamine H_3 receptor led to inhibition of histamine synthesis and decreased release of histamine from neuronal synaptic vesicles (Garbarg et al. 1989). Specific agonists (i.e. *R*-α-methylhistamine) and antagonists, such as thioperamide, were available soon after the description of this new receptor (Arrang et al. 1987).

Histamine H_3 receptors were also shown to behave as heteroreceptors, modulating the release of several neurotransmitters such as noradrenaline (Schlicker et al. 1994), acetylcholine (Vollinga et al. 1992), serotonin (Schlicker et al. 1988), dopamine (Schlicker et al. 1993) and neuropeptides from nonadrenergic noncholinergic nerves (Burgaud et al. 1993). Taking into account the multiple roles of those neurotransmitters and the presence of histamine H_3 receptors in several areas of the central nervous system (CNS), such as the ventral striatum, substantia nigra and hypothalamus (Ligneau et al. 1994), the use of histamine H_3

C. Pérez-García · L. Morales · M.V. Cano · I. Sancho
L.F. Alguacil (✉)
Laboratory of Pharmacology,
University of San Pablo CEU, P.O. Box 67,
E-28660 Boadilla, Madrid, Spain
e-mail: laguacil@ceu.es, Fax: +34-1-3510475

receptor ligands could be expected to have therapeutic potential in the treatment of different CNS disorders. At present, however, neither the psychopharmacology of histamine H_3 receptors nor the therapeutic value of their ligands is clear enough. The aim of this work is to study these questions further by investigating the possible involvement of histamine H_3 receptor function in experimental models of anxiety and depression.

Histamine H_3 receptors have been thought to play a role in sleeping (Monti et al. 1993) and convulsions (Yokoyama et al. 1994). These processes are also modulated by anxiolytic compounds of the benzodiazepine type; accordingly, it would be advisable to check the effect of histamine H_3 receptor ligands in models of anxiety such as animal behaviour in the elevated plus maze.

It has been suggested that the central histaminergic system may be involved in the effects of antidepressants (Schwartz et al. 1981). Furthermore, histamine-induced catalepsy has been proposed as a model for the evaluation of antidepressant drugs (Onodera et al. 1991). The effect of some antidepressants could be mainly related to modifications of histaminergic mechanisms. Thus, levoprotyline-induced decrease of immobility time in the forced swimming test has been directly related to its action as a histamine H_1 receptor antagonist (Noguchi et al. 1992). The possible involvement of histamine H_3 receptor function in stress and depression has been previously studied by Ghi et al. (1995a,b), who showed that the tricyclic antidepressant amitriptyline counteracted the stress-induced decrease of histamine H_3 receptor density in rat brain cortex and increased the number of receptors in non-stressed animals. Lamberti et al. (1998) have recently found that thioperamide exhibited an antidepressant-like activity in the mouse forced swimming test, but their results could not rule out a possible contribution of increased locomotor activity to the effect observed (a common cause of false positive results in this test); moreover, a possible action of thioperamide on other pharmacological targets such as 5-HT_3 receptors (Leurs et al. 1995) could be involved. We have tried to verify the possible effect of the histamine H_3 receptor blockade on mouse forced swimming by running parallel experiments with thioperamide in a locomotor activity paradigm. Furthermore, the effect of another histamine H_3 receptor antagonist, clobenpropit, has been studied in the same experimental conditions. Preliminary results were presented at the VII Meeting of the Spanish Society of Neuroscience (Pérez-García et al. 1997).

Materials and methods

Elevated plus-maze test

Animals

Male Sprague-Dawley rats (180–220 g; San Pablo-CEU University bred) were housed under a 12:12 dark/light cycle (lights on at 8:00 a.m.), at an ambient temperature of 22 ± 1°C and fed with standard diet. Two hours before the test the animals were moved to the experimental room where they had free access to food and water.

Drugs

Diazepam (2 mg/kg; Valium®, Roche, Spain), *R*-α-methylhistamine dihydrochloride (10 mg/kg; RBI, USA) and thioperamide maleate (0.2, 2 and 10 mg/kg; RBI, USA) were dissolved in physiological saline and injected IP in a volume of 10 ml/kg, 30 min before the test. Control injection consisted of an equivalent volume of saline.

Apparatus and procedure

The elevated plus maze was made of black Plexiglas and consisted of two opposing open arms (50 × 10 cm^2) and two enclosed arms (50 × 10 × 40 cm^3) united by a central platform (10 × 10 cm^2) and elevated to a height of 60 cm above the floor level. The experimental room was lit by red fluorescent lamps.

For behavioural testing, the rats were placed individually on the central platform facing a closed arm and allowed 5 min of free exploration. The behaviour was recorded by a videocamera linked to a videotracking system (San Diego Instruments, USA) which automatically quantified the time spent in the open arms. The tests were always run between 1400 and 1900 hours.

Forced swimming test

Animals

Male OF1 mice (IFFA-CREDO) weighing 25–30 g were used. Animals were kept at 22 ± 1°C, with commercial diet and tap water ad libitum and under a 12:12 dark/light cycle.

Drugs

Imipramine (10 and 15 mg/kg; Sigma, Spain), *R*-α-methylhistamine dihydrochloride (10 and 20 mg/kg; RBI, USA), thioperamide maleate (0.2, 2, 10 and 20 mg/kg; RBI, USA) and clobenpropit dihydrobromide (5 mg/kg; RBI, USA) were dissolved in physiological saline and injected in a volume of 10 ml/kg, 60 min prior to the test. Control animals received an equivalent volume of saline.

Procedure

Animals were individually introduced for 6 min in a glass cylinder (11 cm in diameter and 14 cm in height), which was filled to a depth of 7.5 cm with water maintained at 25°C. The immobility time of each animal was measured during the last 4 min.

Locomotor activity

Mice (as used in the forced swimming test) were placed in individual cages (25 × 25 × 14 cm^3) and allowed to habituate themselves to the novel environment for 30 min. The animals were then treated (IP 10 ml/kg) with saline, thioperamide maleate (10 mg/kg), clobenpropit dihydrobromide (5 mg/kg) or amphetamine sulphate (3 mg/kg) and immediately returned to the cages. The locomotor activity of the animals was continuously recorded for 90 min by a

BNSDOCID: <XP 980226A 1 >

Table 1 Effect of thioperamide and *R*-α-methylhistamine on the time spent in the open arms of the elevated plus maze (*$P < 0.05$ with respect to saline)

Treatment	*n*	Time spent in open arms (s) mean ± SEM	% Change with respect to saline
Saline	32	32.55 ± 4.50	-
Diazepam 2 mg/kg	10	96.79 ± 35.95*	197.30
R-methylhistamine 10 mg/kg	12	61.04 ± 14.52	87.53
Thioperamide 0.2 mg/kg	7	55.48 ± 19.59	70.44
Thioperamide 2 mg/kg	11	18.07 ± 8.85	−44.48
Thioperamide 10 mg/kg	8	35.74 ± 6.39	9.8

Table 2 Effect of thioperamide and *R*-α-methylhistamine on the immobility time in the forced swimming test (*$P < 0.05$ with respect to saline)

Treatment	*n*	Immobility time (s) mean ± SEM
Trial 1		
Saline	11	150.10 ± 10.22
Imipramine 15 mg/kg	11	89.36 ± 8.36*
Thioperamide 10 mg/kg	11	100.91 ± 9.78*
Trial 2		
Saline	21	166.24 ± 6.48
Imipramine 10 mg/kg	11	130.54 ± 12.68*
Thioperamide 0.2 mg/kg	10	160.00 ± 9.90
Thioperamide 2 mg/kg	10	142.30 ± 29.78
Trial 3		
Saline	26	166.27 ± 9.68
Thioperamide 20 mg/kg	10	144.70 ± 13.78
R-α-methylhistamine 10 mg/kg	10	159.80 ± 12.18
R-α-methylhistamine 20 mg/kg	17	180.53 ± 12.15

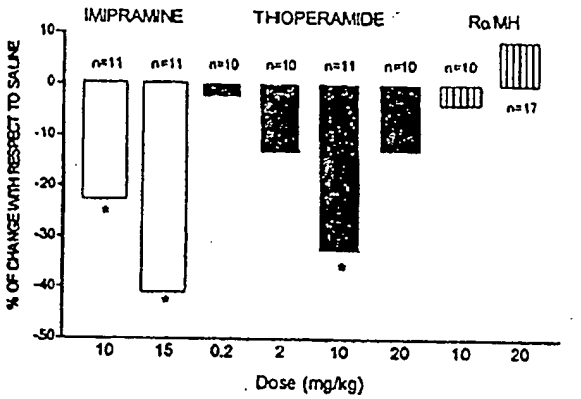

Fig. 1 Forced swimming test. Effect of thioperamide (0.2, 2, 10 and 20 mg/kg) and *R*-α-methylhistamine (*RαMH*; 10 and 20 mg/kg) on the immobility time. Imipramine (10 and 15 mg/kg) was used as a positive control (*$P < 0.05$ with respect to saline)

magnetic activity meter (Panlab, Spain) placed at the bottom of the cages. This device transformed locomotion-induced changes of magnetic field into arbitrary activity counts.

Statistics

Results of experiments were analysed by one-way analysis of variance (ANOVA) followed by Newman Keuls post-hoc test. Significance was considered at the 0.05 level.

Results

Results of the elevated plus maze test are given in Table 1. Intraperitoneal administration of diazepam (2 mg/kg) resulted in a significant increase in the time spent in the open arms, while neither thioperamide nor *R*-α-methylhistamine significantly changed this variable.

In the forced swimming test, thioperamide dose-dependently decreased the immobility time. The reduction was significant at 10 mg/kg and intermediate between that of the two doses of imipramine assayed. A higher dose of thioperamide (20 mg/kg) elicited a lower, non-significant reduction of immobility. The behaviour of mice treated with *R*-α-methylhistamine was similar to that of control mice, resulting in the lack of significant differences concerning immobility times in these cases. The results of three independent trials are shown in Table 2 and are globally represented in Fig. 1.

In the activity meter, locomotor activity was significantly increased by amphetamine 60 and 90 min after administration and was not modified by thioperamide at any time (Fig. 2A). Figure 2B shows the locomotor activity exhibited by mice from 60 to 66 min postinjection, which coincides with the time interval used in the forced swimming test; as above, thioperamide failed to affect locomotor activity in this interval.

Clobenpropit was also tested for its effects on swimming behaviour and locomotion. As in the case of thioperamide, this drug decreased immobility in the forced swimming test but did not significantly modify animal performance in the activity meter (Table 3, Fig. 3).

Discussion

Results from the elevated plus maze show that, at the doses tested, neither *R*-α-methylhistamine nor thioperamide affected the time that animals spent on the open arms. Since this variable is increased by drugs with anxiolytic activity, these results allow us to suggest that neither the stimulation nor the blockade of

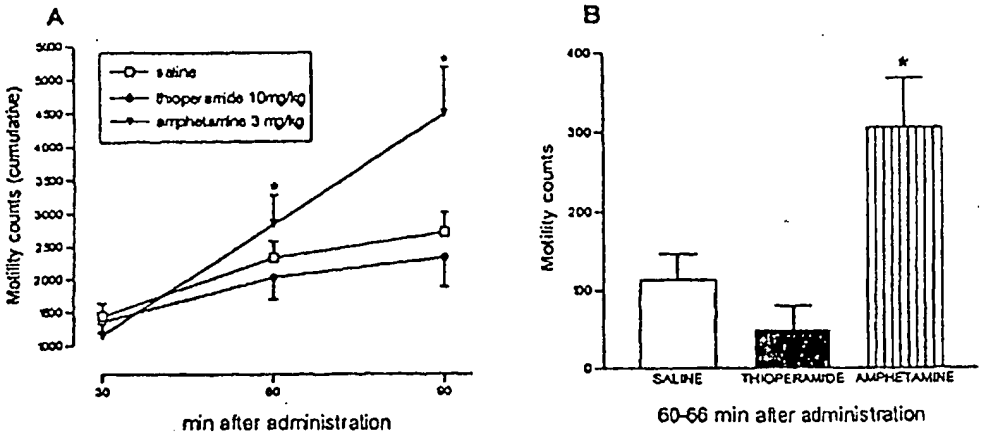

Fig. 2A, B Effect of thioperamide (10 mg/kg) and amphetamine (3 mg/kg) on the locomotor activity measured in an activity meter. Results are means ± SEM ($n = 11$) (*$P < 0.05$ with respect to saline)

Table 3 Effect of clobenpropit on the immobility time in the forced swimming test (*$P < 0.05$ with respect to saline)

Treatment	n	Immobility time (s) mean ± SEM	% Of change with respect to saline
Saline	12	160.30 ± 7.73	
Imipramine 15 mg/kg	12	88.33 ± 11.69*	−44.90
Clobenpropit 5 mg/kg	12	121.00 ± 12.11*	−24.52

histamine H_3 receptors elicits a change in the level of anxiety. However, the predictive value of the elevated plus maze has some limitations, i.e. it fails to detect the anxiolytic effects of partial 5-HT_{1A} agonists such as buspirone (Dawson and Tricklenbank 1995). Therefore, it would be advisable to test *R*-α-methylhistamine and thioperamide in more robust models of anxiety.

The results obtained in the forced swimming test increase previous evidence of a possible antidepressant effect of thioperamide (Pérez-García et al. 1997; Lamberti et al. 1998). Blockade of histamine H_3 receptors seems directly involved, since clobenpropit, another histamine H_3 receptor antagonist, is also active in the test. The administration of thioperamide reduced the immobility time in a dose-dependent manner, significant at 10 mg/kg. The results from the activity meter tend to discard hyperactivity as the mechanism responsible for the effect obtained. This variable was not considered by Lamberti et al. (1998) in their studies, in spite of the fact that psychostimulation can lead to false positive results in the forced swimming test (Borsini and Meli 1988). In fact, these authors combined the forced swimming test with the study of rotarod performance, which detects changes of motor coordination but not necessarily stimulation. Previous findings from other authors also showed that thioperamide (10 mg/kg) does not increase locomotor activity in mice: by using a dose range from 0.2 to 10 mg/kg, Clapham and Kilkpatrick (1994) showed that the histamine H_3 receptor antagonist did not increase motor activity and even counteracted stimulant-induced hyperactivity. Thioperamide was only found to increase locomotor activity in mast-deficient mice at higher doses (20 mg/kg) (Sakai et al. 1991), which were devoid of significant effects in the forced swimming test.

The finding that thioperamide produces no significant effects in the forced swimming test at 20 mg/kg while being active at lower doses is consistent with the results of Lamberti et al. (1998) in this test, and also with other data from the literature. Chiechio et al. (1997) reported that thioperamide at 10 mg/kg provokes hyperalgesia and inhibits clonidine analgesia in tail-flick and hot-plate tests; at 15 mg/kg, it is less active on both parameters and at 25 mg/kg, it is analgesic and enhances the effect of clonidine. Furthermore, in the study of Sakai et al. (1991), low doses of thioperamide were found to increase locomotor activity of mast-deficient mice, whilst higher doses reduced locomotor activity and impaired motor coordination. It is possible that high doses of thioperamide could produce pharmacological effects independent of the histamine H_3 receptor blockade which could interfere with the more specific actions, i.e. 5-HT_3 antagonism (Leurs et al. 1995). However, a similar biphasic effect has been reported when the action of the H_3 receptor agonist *R*-α-methylhistamine on leptazol-induced convulsions was studied (Sturman et al. 1994). Therefore, it is more likely that H_3 receptor function could be directly involved in these biphasic effects of both agonists and antagonists. Adaptation of receptors to excessive blockade or stimulation with high doses of their ligands may also account for these results; in fact, rapid adaptations of H_3 receptors have been described in peripheral tissues (Pérez-García et al. 1998). Competition between histamine H_3 receptor ligands and endogenous-released histamine could also contribute to these biphasic effects, as suggested by Lamberti et al. (1998).

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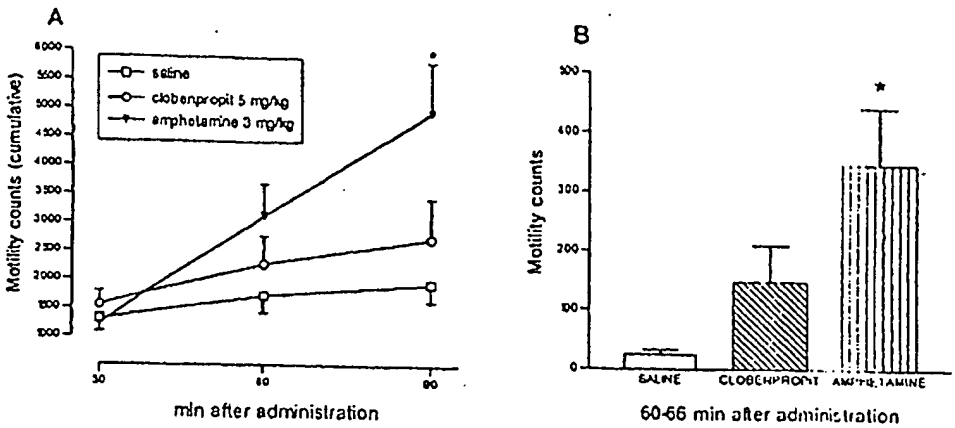

Fig. 3A, B Effect of clobenpropit (5 mg/kg) and amphetamine (3 mg/kg) on the locomotor activity measured in an activity meter. Results are means ± SEM ($n = 10$) (*$P < 0.05$ with respect to saline)

The mechanism through which thioperamide exerts its action in the forced swimming test cannot be explained easily by a H_3-mediated increase of histamine release. Lamberti et al. (1998) have suggested that thioperamide could reduce immobility by this mechanism; thus, the histamine released would be directly responsible for the antidepressant-like action by stimulating histamine H_1 postsynaptic receptors. Although these authors obtained a dose-dependent reduction of immobility with histamine H_1 receptor agonists, it should be pointed out that some data from the literature are not in agreement with this idea. Noguchi et al. (1992) have found that forced swimming increases histamine function and that histamine H_1 receptor antagonists such as mepyramine reduce immobility, which is just the opposite to the findings of Lamberti et al. (1998). In another model of depression, the olfactory bulbectomized rat, the histamine H_1 receptor antagonist terfenadine also showed an antidepressant-like effect (Song et al. 1996). Alternatively, the putative antidepressant properties of thioperamide may reside in a histamine H_3 heteroreceptor-mediated stimulation of the release of other neurotransmitters, mainly on its proved ability to increase the central levels of noradrenaline (Schlicker et al. 1994) and serotonin (Schlicker et al. 1988). As is well known, antidepressants like fluoxetin block serotonin uptake, whereas tricyclic antidepressants inhibit both serotonin and noradrenaline uptake (Baldessarini 1996).

In summary, the results from the forced swimming test indicate a possible antidepressant activity of thioperamide at 10 mg/kg, whereas *R*-α-methylhistamine did not show any significant effect. None of those histamine H_3 receptor ligands seem to modify the state of anxiety in the elevated plus maze. Further experiments using other models of depression and anxiety will be needed to confirm these findings; in the case of forced swimming, we have discarded a false positive result due to psychostimulation, but it must be pointed out that a significant number of other drugs with quite different pharmacological properties (i.e. clozapine, diphenylhydantoin, cyproheptadine, ouabain, etc.) are also active in behavioural despair paradigms (Borsini and Meli 1988).

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Transgenic knockouts as part of high-throughput, evidence-based target selection and validation strategies

Stephen Harris

The worldwide genome sequencing projects are helping to define the size and complexity of the expressed genome and are thereby identifying an unprecedented number of genes of uncertain disease alignment and unknown function. It is widely recognized that, within the pharmaceutical industry, a significant commercial advantage will accrue to those companies that most effectively gather and integrate additional biological information into their therapeutic target selection and drug progression strategies. This article presents the rationale for including comparative phenotypic information obtained from transgenic gene knockouts as an integral part of any future therapeutic target selection strategy.

Stephen Harris
Technical Evaluation (Europe)
GlaxoSmithKline Research & Development
Medicines Research Centre
Gunnels Wood Road
Stevenage
UK SG1 2NY
tel: +44 1438 764964
fax: +44 1438 764810

The completion of the first draft of the human genome sequence is, with the other ongoing genome sequencing projects, helping to define the size and complexity of the expressed mammalian genome, including an unprecedented number of genes of uncertain disease alignment and unknown function. As all gene sequences represent potential therapeutic targets, strategies must be developed to gain the additional biological insight that allows rate-limited R&D resources to be focused on those genes with the greatest therapeutic, and thereby commercial, potential. The emphasis within exploratory research is therefore shifting towards the evaluation and adoption of high-throughput technology platforms that can add additional value to the gene selection process, through functional studies or other measures of disease alignment, including genetics, differential gene expression, proteomics, tissue distribution, comparative species data and so on. The competition to achieve this will be particularly intense for those additional candidate gene family members that currently represent the chemically tractable or 'drugable' gene targets[1–4].

The potential of phenotypic information derived from gene knockouts to contribute to a high-throughput target selection/validation strategy has hitherto been limited by the resources required to generate and characterize a large number of gene knockout transgenics. Recently, several biotechnology companies have set out to address these issues and thereby to create an opportunity for the pharmaceutical industry to access this information in a timely fashion and on a previously unprecedented scale[5]. In this article, these opportunities are assessed with respect to the strategic business needs and changing organizational models being adopted within the pharmaceutical industry.

Therapeutic target selection: key considerations

The pharmaceutical industry is primarily focused on those key diseases for which there is a significant unmet clinical need and thereby future commercial value. To achieve this, the overall drug discovery process can be viewed as a series of key milestones or checkpoints at which go/no-go decisions are made about proceeding with a portfolio of projects. These decisions are based on the available evidence that individual projects have attained a minimum set of progression criteria. Until recently, maximizing diversity within HTS and the properties of compounds in preclinical and clinical settings has been a primary focus within the industry. This trend has been a result of, in part at least, the fact that the steady (rather than spectacular) rate of biological target identification and validation has meant that the portfolio of therapeutic targets has been relatively small. As such, the

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physiochemical properties of a therapeutic product are particularly important to market success[6].

With the emergence of the human genome sequence, companies are increasingly beginning to focus on developing target selection strategies that will aggressively collect and integrate biological data into therapeutic target selection decisions, especially for those candidate genes considered to be most likely to deliver value to the business. To prosecute such a strategy, and thereby to minimize the overall R&D risk inherent in the initial choice of a therapeutic target from within a candidate gene pool, a pragmatic set of evidence-based selection criteria are needed upon which to base go/no-go decisions. Ideally, the outcome of such a shift in strategic emphasis should be an exploratory discovery organization capable of delivering a sustainable output of highly validated molecular targets into drug discovery[1].

In the short-to-medium term, therapeutic target selection decisions within exploratory research organizations are likely to become focused on two key properties of a candidate gene (Fig. 1). In the simplest terms, these can be portrayed in two dimensions as the relative positions of a portfolio of individual candidate genes along 'therapeutic' and 'chemical space' axes. In this model, the 'chemical space' axis represents the relative probability of obtaining viable chemical entities for progression within the drug discovery process after screening against the candidate gene, an estimate based on the historical precedent for the target class in question. The therapeutic axis represents the relative strength of the evidence for a disease association (or scientific rationale) between a given candidate gene and the desired therapeutic and/or mechanistic profiles of the disease of interest[6].

At a given time, there are likely to be exploratory projects, including multiple candidate genes, occupying points throughout this two-dimensional matrix, each seeking to gather evidence that results in a candidate gene achieving the relevant target selection threshold and thereby progressing into the drug discovery pipeline. As a natural consequence of using these criteria, most projects progressing from exploratory discovery into the drug discovery pipeline are likely to become focused on genes from within the chemically tractable families for which there is greatest evidence of a disease association (Zone A, Fig. 1). In addition, a limited number of pathway expansion projects focused on biological mechanisms with a strong scientific rationale (e.g. genetic, clinical or functional correlation with a disease phenotype) are likely to be pursued to identify a chemically tractable gene as a point of therapeutic intervention (Zone B, Fig. 1). These two axes are considered in more detail below with particular reference

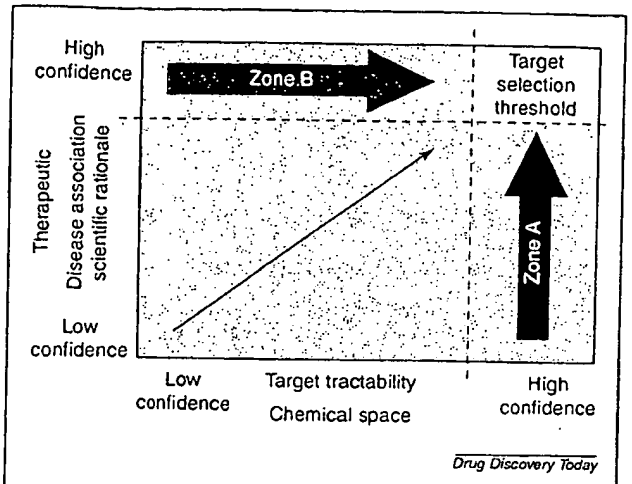

Figure 1. Key components of therapeutic target selection. In order to minimize the overall R&D risk inherent in the initial choice of a therapeutic target, such decisions often need to focus on two key properties of individual members of any pool of candidate genes. In the simplest terms, these can be described as (1) the 'therapeutic' axis, or the strength of the disease association (or scientific rationale) between a candidate gene and the disease of interest, and (2) the 'chemical space' axis, or the gene product's innate chemical tractability or 'drugability', an estimate based on historical precedent. Ideally, at any one point in time, the portfolio of therapeutic candidate genes moving from exploratory research into the drug discovery pipeline should be the subset of any pool of candidate genes attaining at least the minimum target selection thresholds established for each of these axes.

to the potential role of gene knockout phenotypes in helping to define the therapeutic alignment of individual members of the chemically tractable classes of candidate gene.

Bioinformatics: defining candidate gene targets

The draft human genomic sequence[7] is being intensively analyzed by bioinformaticians throughout the world to identify and catalogue all the genes within the expressed human genome. The uncertainty inherent in this task is illustrated by the broad range of values that had previously been estimated for the overall size of the expressed human genome, ranging from 28,000 up to >120,000 genes[8,9]. Although the final number is more likely to be in the range 30,000-40,000, this will no doubt continue to engender informed debate and good-humoured speculation (http://www.ensembl.org/) up until, and even beyond, the completion of the final draft of the human genome sequence, scheduled for 2003.

For the pharmaceutical industry, this represents a once-in-a-lifetime opportunity to participate in determining the size and complexity of the human candidate gene pool that

will contain all future therapeutic targets. More importantly, in the short term, this exercise will help define all the novel members of the gene families that are currently known to be chemically tractable or drugable, based on historical precedent (i.e. they are within Zone A, Fig. 1). Based on an extrapolation of the current therapeutic target classes, this subset of the genome is estimated at some 5000–10,000 genes, representing the G-protein-coupled receptors (GPCRs), ion channels, proteases, kinases and so on[1,6].

The potential value attributed to this DNA sequencing and data-mining activity is illustrated by the significant effort that has been expended in seeking a commercial advantage through development of a portfolio of gene-based intellectual property (IP)[10]. Although the future commercial value of the first phase of DNA-sequence-based IP remains uncertain, further biological insight around disease alignment and/or function might result in 'reach-through' claims that undermine the traditional commercial value of third-party chemical IP established around the biological target. In the short-to-medium term, therefore, companies that fail to establish biological IP (alone or in partnership) within the chemically tractable therapeutic gene-families might either be blocked from working on key molecular targets aligned to disease or obliged to pay significant royalties and/or milestone fees to third parties.

Despite significant efforts directed at understanding the biological significance of the expressed genome throughout the academic and commercial worlds, there is still an enormous number of genes of uncertain disease relevance and unknown function. Bioinformaticians can only infer biological function for the vast majority of genes, at least until additional biological annotation is deposited within public and proprietary databases[9]. The biological data that contribute to an increased level of confidence (validation) of any candidate gene on the therapeutic axis (Fig. 1) will come from a diverse set of activities, not all of which will be applicable to every disease state.

In the future, bioinformatics will, therefore, play an increasingly crucial role not only in continuing to identify and catalogue the chemically tractable genes within the genome but also in supporting the capture, integration and mining of gene-based biological annotation from a diverse set of experimental paradigms. Organizations that develop efficient knowledge management capabilities, as an integral part of any high-throughput biology effort, will benefit most from any shift in emphasis to a genomics-based target selection strategy.

Disease-based and gene-based approaches

In response to the opportunity and challenge represented by the scale of the expressed genome, the emphasis is beginning to shift from predominantly disease-to-target to gene-to-target strategies (Fig. 2). At this point, it is essential not to lose sight of the fact that both strategies are crucially dependent upon, and sensitive to, continued improvements in our background understanding of disease phenotypes and associated mechanisms and, therefore, need to be flexible enough to adapt to new knowledge. They are also not mutually exclusive strategies; rather they represent two extremes, both of which will already exist in a state of dynamic equilibrium within many pharmaceutical companies. There is, however, a widespread recognition of the need to adopt organizational models and processes aimed at streamlining the larger-scale collection and evaluation of biological information, including significant outsourcing options[2–4].

The disease-orientated strategy has historically favoured an exploratory research organization built around disease-focused multidisciplinary teams seeking to identify and validate the role of 'novel' candidate genes within the current understanding of the disease, and in clinical practice, cellular systems and comparative species (Fig. 2a). In this model, disease knowledge shapes the source of candidate genes and the crucial path of a portfolio of exploratory research projects moving outward from the most validated point(s) in biological space. This strategy classically involves repeated cycles of gene identification and functional validation (often partial), followed by HTS and drug development, with each cycle tailored to the hypothesized properties of the most current 'nearest-neighbour' set of candidate genes. This approach explains, at least in part, the consolidation of pharmaceutical interest around a similar set of clinically precedented or biologically well-validated therapeutic targets and/or pathways. As part of such a disease-orientated model, scarce resources to create and characterize gene knockouts are generally, but not exclusively, focused on supporting target validation of a prioritized shortlist of disease-aligned candidate genes, rather than driving target selection decisions *per se*.

By contrast, the gene-orientated strategy assumes the same background disease knowledge as above but favours an exploratory research organization built around a suite of technology platforms focused on high-throughput delivery of the key comparative biological data required to select individual targets from any pool of candidate genes (Fig. 2b). In this model, the prioritization of therapeutic targets occurs after systematically generating the minimum set of comparative biological properties that any pool of candidate genes must exhibit to meet the target selection threshold defined for the disease of interest, and thereby progress to drug discovery. The option to incorporate comparative phenotypic information derived from gene

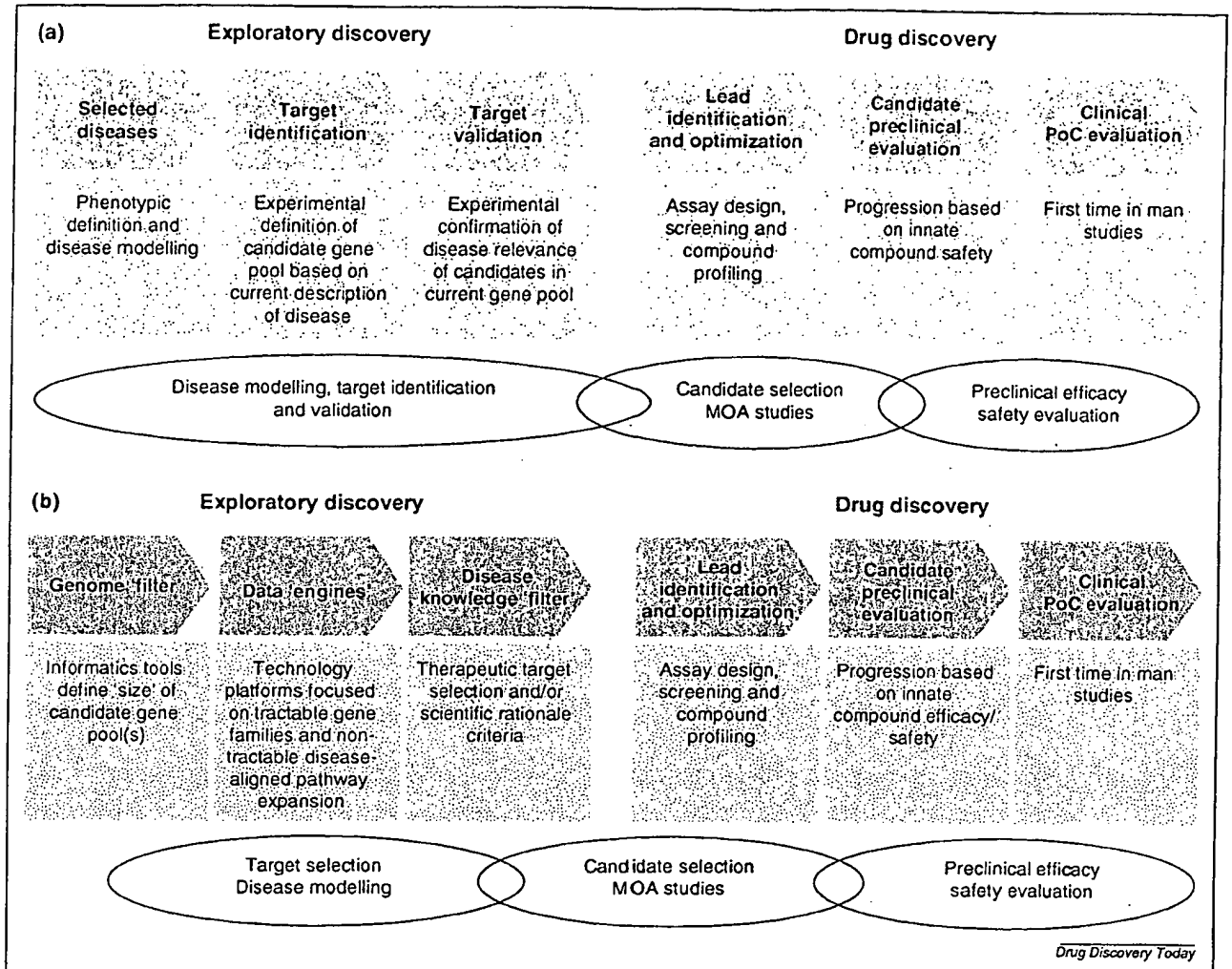

Figure 2. Therapeutic target selection: disease-based and gene-based approaches. Information obtained from transgenic gene knockouts can be used in decision making at various points within the overall pharmaceutical discovery process, several stages of which are illustrated here. The greatest effect of the timely delivery of comparative phenotypic information from gene knockouts will be within exploratory discovery, where the selection and validation of therapeutic targets from within the portfolio of chemically tractable candidate genes is becoming increasingly important. The gene-knockout animals, tissues or cells derived from them might also prove useful at several other stages of the drug discovery process. Abbreviations: MOA, mode of action; PoC, proof of concept.

knockouts into this strategy has, until recently at least, been limited by the resources required to systematically generate and characterize a large number of gene knockout transgenics in a timely manner[5] (discussed also later).

Attributing a relative value to biological data

Faced with a portfolio of 5000–10,000 chemically tractable candidate genes, there are few, if any, technology platforms that can provide definitive biological evidence that a particular candidate gene, or subset of candidate genes, are therapeutically relevant in a systematic or timely fashion[2,6]. The industry is, therefore, in a transition phase in which it is currently obliged to make a series of pragmatic, intermediate target-selection decisions based on incomplete biological information, while continuing to seek access to the comparative biological information it needs to make better informed decisions in the future. Based on historical precedent, these biological data filters fall into three categories, or combinations thereof, that broadly reflect their relative value to the overall therapeutic target-selection threshold depicted in Fig. 1: (1) expression studies, (2) *in vitro* and *ex vivo* functional studies and (3) molecular genetics and *in vivo* studies. Moreover, biological insight gained from the human population, or material sourced from the human

population, has the highest value but data from one or more comparative species are often used as a surrogate in exploratory biology[2,5,6].

Gene expression studies

Gene expression profiling has a relatively high throughput and a low to medium value, and is often used as a 'first-pass' means of defining a subset of genes with an appropriate tissue and/or cellular distribution pattern considered relevant to the pathophysiology of the disease of interest. Array-based transcriptional profiling[11] and proteomics[12] are complementary approaches that are radically changing the way in which such expression studies are conducted. Both generate significant amounts of information about steady-state expression levels and/or changes in expression profile under differing conditions (e.g. diseased and undiseased tissue, treated and untreated cells). Using these approaches, not only can changes in expression profile be observed but also some degree of functional validation can be inferred, given appropriate experimental design and a suitable framework of prior art. The highly parallel nature of these platforms can, however, result in large volumes of data, which in itself can be a challenge when considering which candidate genes to take forward for further validation.

In vitro *and* ex vivo *functional studies*

There are several *in vitro* and *ex vivo* approaches for performing functional studies with low to medium throughput and medium to high value. They are based on well-established techniques such as the yeast two-hybrid system, cloning by complementation and expression cloning and so on. They all provide a means of identifying and/or validating a candidate therapeutic target, usually within a defined cellular or biochemical context. The challenge will be designing and scaling these mechanism-based approaches and combining them with gene-modulation tools such as antisense oligonucleotides[13] to directly assess the functional contribution of candidate genes from within the chemically tractable target classes. In the long term, these types of studies will contribute to intracellular 'pathway maps', especially when combined with expression profiling techniques. These maps will allow researchers rapidly to make biological connections between disease-aligned intractable candidate genes to those chemically tractable genes that are most likely to be developed into products with a therapeutic effect.

Molecular genetics and in vivo *studies*

Molecular genetics and *in vivo* studies have a low throughput and a medium to high value. Population genetics in both humans and comparative species has a proven track record of unambiguously identifying genes responsible for particular disease phenotypes[14–16]. Although this success has, in the main, been restricted to monogenic disorders, recent advances in this area are beginning to extend the utility of this approach to the more common polygenic disease states. Rodents, especially the mouse, are expected to play an increasingly significant role in determining the functional significance that specific genes play in complex diseases states[5].

Although molecular genetics provides a high degree of confidence that a particular gene is responsible for a specific disease state, there is a relatively low probability (~0.1) that the gene will be a member of a chemically tractable gene family. Furthermore, it is proving to be a significant challenge to translate a genetically defined but chemically intractable gene of limited or unknown function into a chemically tractable therapeutic target that is amenable to drug discovery (i.e. to cross Zone B, Fig. 1). In many organizations, the resources available to gain the additional biological understanding required to progress with a novel genetically defined candidate gene or pathway within exploratory research are limited, relative to the study of chemically tractable candidate genes.

When considering how best to address the challenge of gathering sufficient biological evidence to allow comparative, evidence-based therapeutic target selection decisions, the industry is, therefore, faced with some difficult, and often expensive, choices. The scientific rationale and business assessment (principally the risk:return ratio) leading to any portfolio of investments in a high-throughput biology capability has, ultimately, to be a matter of individual organizational judgement based on several factors:

- The perceived value that an individual biological observation makes to the comparative therapeutic target selection process.
- An assessment of the likelihood that a sufficient proportion of all such observations will be of similar value, thereby enabling candidate genes to be prioritized as part of a comparative, evidence-based therapeutic target selection strategy.
- The perceived returns justify the overall cost of gaining access to the information or tools.

Functional genomics and transgenic gene knockouts

The selection of biological targets for the development of potential new medicines relies, in part, on the quality of the *in vivo* biological data that relates a particular molecular target with the underlying pathophysiology of a disease. Since the late 1970s, several techniques have been developed that allow the production of transgenic animals with defined genome modifications, and so the mouse has increasingly become the species of choice for mammalian

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gene function studies (Box 1). Within the pharmaceutical industry, transgenic animals, especially gene knockouts, are proving to be invaluable sources of functional information and tools that can be used in studies at various other stages of the drug discovery process (Fig. 2). For example, in preclinical candidate drug selection, information obtained from gene knockout and/or gene addition transgenics is increasingly being accepted as a viable cost-effective alternative for mutagenicity and carcinogenicity testing[17,18]. In addition, where relatively imprecise pharmacological reagents are available, gene knockouts can be used to define the biological mode of action by helping to discriminate between the *in vivo* gene function(s) of closely related members of a gene family[19–22]. These few examples illustrate the potential power of comparative studies when the appropriate gene knockout reagents are available.

The most significant impact of transgenics is currently in the exploratory phase, where gene knockouts are predominantly, but not exclusively, created to support target validation as part of a disease-to-target strategy (Fig. 2a). The manipulation of gene function *in vivo* can provide a high degree of confidence that the gene of interest is a crucial component of the biology under investigation and thereby help to focus scarce resources on progressing candidates that exhibit the phenotype of greatest clinical relevance (Box 2). The potential of gene knockout transgenics to contribute to high-throughput target selection and/or validation has hitherto been limited by their availability, and/or the resources required to generate and characterize a large number of gene knockouts.

Box 1. Generating and characterizing transgenic animals

Transgenic animals are commonly generated either by pronuclear DNA microinjection[a] or by gene targeting via homologous recombination in embryonic stem cells[b]. The recent demonstration that gene targeting can be performed in sheep means that targeted gene modification might become routinely available in other species[c–e]. Although there are several theoretical and practical caveats and limitations associated with using gene addition and gene knockout transgenics in functional analysis, including copy number, site of integration effects, embryonic lethality and genetic background effects, the continued development and adoption of conditional knockout and knock-in approaches, along with other techniques, will probably provide the opportunity to overcome some of these limitations and thereby obtain further mechanistic insights into *in vivo* gene function[f–l].

In the context of exploratory drug discovery, the success of this technology platform for target selection ultimately depends on the phenotypic description of the gene knockout used to make a gene-to-disease correlation. A serious 'phenotyping gap' is emerging that is, in part at least, a result of the practical considerations inherent in establishing the breadth and depth of first-pass analysis currently used in the high-throughput phenotypic screening of both chemically induced mutants and gene knockouts[m,n]. For some diseases, it will be essential, albeit challenging, to invest in the secondary and tertiary phenotypic screens, including aging studies, that will be required to build confidence in comparative target selection in certain therapeutic areas.

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Target selection using large-scale knockout phenotyping

The value (impact) of a gene knockout phenotype on the target selection and candidate drug progression process can be assessed by examining how the phenotypic information derived from gene knockouts has been historically used in decision making within the drug discovery process. Although this exercise can be performed for any pool of candidate genes, the example used here summarizes the conclusions for one class of chemically tractable gene, the gene family encoding the GPCRs (Ref. 5). An internal survey of discovery projects revealed that, if gene knockout information were readily available, up to ~25% of all non-olfactory *GPCR* gene knockout phenotypes could contribute significantly to the scientific rationale leading to the selection of a *GPCR* family member as a therapeutic target for drug discovery. A further ~60% of gene knockout phenotypes could potentially help to discriminate between candidate genes within a gene family whereas 10–15% of

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Box 2. Case study: obesity phenotype, gene knockouts and therapeutic target selection

Obesity is becoming an increasingly important health problem around the world, not least because it causes or exacerbates other disease states. Remarkable progress has been made recently in understanding body-weight regulation through studies of this phenotype in the mouse. These mechanistic insights into the complex pathophysiology of this disease have been gained using both molecular genetic approaches and targeted gene knockouts, and are, in some cases at least, being complemented by studies in humans[a].

More specifically, the phenotypes of knockout mice lacking genes encoding components of the melanocortin system have highlighted the fact that specific members of the G-protein-coupled melanocortin receptor (MCR) family are involved in regulating body weight through distinct and complementary mechanisms[b]. Moreover, within this five-member gene family, the available gene knockouts have helped to distinguish distinct functional roles for the *MCR-3/4* and *MCR-5* genes in spontaneous obesity and exocrine gland dysfunction, respectively[c–f].

This example illustrates the effect that phenotypes exhibited by gene knockouts might have on therapeutic target selection. That is, the primary output of a comparatively high-throughput knockout phenotyping capability can help to focus rate-limited R&D resources on candidates with the greatest therapeutic and thereby commercial potential. Moreover, it is specifically proposed that, for the pool of chemically tractable genes of uncertain or unknown function within the human genome, accessing comparative *in vivo* phenotypic information from gene knockouts will provide a competitive advantage if incorporated within future therapeutic target selection and validation strategies within the pharmaceutical industry.

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gene knockout phenotypes would initially be misleading in the absence of additional biological information.

This sample set is small and inherently biased towards those gene family members with significant prior art, including pharmacological reagents and/or natural ligands. The analysis was, therefore, extended to include an assessment of the gene knockout phenotypes observed for a total of 63 *GPCR* genes, as described in 111 publications. Approximately, 50% of those gene knockouts examined gave rise to a readily observed phenotype. Of these, ~25% were either overt phenotypes (e.g. obesity, infertility, aggressiveness, embryonic lethality) or phenotypes revealed by a relatively simple experimental measure (e.g. altered pain threshold, haematology, clinical chemistry, behaviour). Approximately 30% of gene knockouts required a more sophisticated experimental challenge before a clinically relevant pathophysiological phenotype was observed (e.g. impaired cellular recruitment in inflammation, altered cognition and memory, glucose intolerance).

These conclusions are dependent upon the level of phenotypic characterization within the literature; for example, a single gene knockout might have exhibited an overt, a simple and/or a complex phenotype and, therefore, contributed more than once to this analysis. Furthermore, based on the experimental hypotheses tested within the literature, ~50% of gene knockouts had no discernible phenotype and, therefore, could not be used to derive a meaningful disease alignment for the gene in question, at least in the absence of a more broadly based or sophisticated battery of phenotypic tests.

These two historical analyses strongly suggested that both the frequency and the value (impact) of comparative gene knockout phenotypes observed for members of the chemically tractable gene families could make a significant contribution to therapeutic target selection, if available at scale and in a timely manner. These general conclusions are highly dependent on the breadth, depth and quality of the comparative phenotypic analysis performed and, in this case, published for each gene knockout. They are also sensitive to the type of phenotypic selection criteria and relative target selection thresholds used in different organizations. Furthermore, for such information to lead to meaningful discrimination between a portfolio of candidate genes, it is important that any decision-making process takes account of any 'negative' phenotype (i.e. does not fit the prevailing scientific rationale) and 'no discernible' phenotype results when prioritizing therapeutic targets.

Accessing a high-throughput knockout phenotyping capability

The key to making comparative evidence-based target selection decisions is the timely delivery of phenotypic information for decision making, rather than the resources to generate the gene knockouts and performing the phenotypic

screens[23]. Many pharmaceutical companies have some in-house transgenic capability or outsource their needs, either via collaboration or to those commercial organizations that generate transgenics on a fee-for-service basis. There is little evidence to suggest that any individual pharmaceutical company is willing to commit their own resources to generate, breed and, most importantly, characterize a large number of gene knockouts in a high-throughput, systematic and timely manner in the near future.

The option of outsourcing phenotypic information from gene knockouts and/or accessing tools, albeit at a reasonable cost, has recently become a reality as biotechnology companies have set out to address the challenges involved in developing a high-throughput gene knockout production and/or phenotyping capability, including Deltagen, Lexicon Genetics and Paradigm Therapeutics. These companies each provide a mechanism by which interested parties can gain access to many candidate gene knockouts and/or the phenotypic information derived from them upon which to make comparative evidence-based target selection decisions. In addition, they offer some type of fee-for-service arrangement whereby potential partners can access specific tools (gene knockout, conditional knockout and knock-in transgenics) for in-house or collaborative phenotypic studies. In this regard, they are similar to several other companies offering transgenic fee-for-service arrangements, albeit seeking to deliver reagents in an accelerated timeframe.

Among other activities, Deltagen (Menlo Park, CA, USA; http://www.deltagen.com/) offers potential partners the option to take out a non-exclusive subscription to Deltabase™. This is a proprietary database that will contain phenotypic information derived from a portfolio of gene knockouts selected from the pool of drugable gene family members. Deltagen has selected >1000 mammalian genes thought to be relevant to small-molecule drug discovery for inclusion in Deltabase. The focus on the 'up-front' delivery of primary comparative phenotypic information for a significant portfolio of gene knockouts of potential interest to the pharmaceutical industry is the distinctive feature of Deltabase. A subscription to Deltabase also provides access to the knockout mice for additional phenotypic studies, as required. The immediate value (impact) of the primary comparative phenotypic information within Deltabase for target selection will depend on how well the breadth and depth of the phenotypic analysis performed by Deltagen aligns with the therapeutic selection criteria of a potential subscriber.

Lexicon Genetics (Woodlands, TX, USA; http://www.lexicon-genetics.com/) is developing Omnibank™, a proprietary sequence database linked to a physical bank of pretargeted mouse embryonic stem cell clones generated by random insertional mutagenesis[24]. This strategy overcomes the need to perform the often time-consuming molecular and cellular biology that is involved in targeting large numbers of candidate genes on a gene-by-gene basis. Once knockout animals have been generated, any phenotypes exhibited by the gene-trap event can be explored either via in-house analysis or using the range of phenotypic screening options Lexicon offers to partners. The Omnibank concept is particularly suited to those interested in gaining access to gene knockout information for potentially any of the expressed genes within the genome. This includes those seeking to validate the functional significance of a portfolio of candidate genes implicated in a priority therapeutic area, or that reside within a defined region of the genome as determined by a genetic approach. More recently, Lexicon has introduced its LexVision™ programme, which closely resembles the Deltabase concept offered by Deltagen. As previously, the impact of the information within the LexVision data set on target selection will depend upon the portfolio of genes analyzed and how well the phenotypic information gathered by Lexicon aligns with the therapeutic selection criteria of a potential subscriber.

Paradigm Therapeutics (Addenbrooke's Hospital site, Cambridge, UK) offers partners access to several transgenic technology platforms. Their aim is to use optimized molecular, cellular and husbandry techniques to generate and discern the phenotypes of gene knockouts for a portfolio of candidate genes from within the drugable gene families, in this case with an emphasis on CNS and metabolic diseases.

Future prospects

The impending fruition of the various genome sequencing projects is helping to define the pool of chemically tractable candidate gene targets and is thereby shifting the emphasis from target identification and validation *per se* to target selection. In the short term, gene-to-target candidate gene selection strategies are likely to have an increasingly significant impact on therapeutic target selection decisions because, by definition, they can be directed towards the chemically tractable classes of gene family. The selection of biological targets for the development of potential new medicines relies, in part, on the quality of the *in vivo* biological data that correlates a particular molecular target with the underlying pathophysiology of a disease. In recent years, transgenic techniques, especially gene targeting, have revolutionized our ability to infer the biological function(s) of genes within an *in vivo* mammalian context.

Within the pharmaceutical industry, the opportunity to use comparative phenotypic information derived from gene knockouts as part of any high-throughput, evidence-based target selection and validation strategy has been limited by availability, either through the literature or by a resource-constrained internal capacity. The emergence of several biotechnology companies specifically focusing on the high-throughput generation and phenotyping of gene knockouts means that the industry now has the opportunity to access this highly informative source of phenotypic information and/or tools in a timely fashion on a previously unprecedented scale. The portfolio of gene knockouts and the breadth and depth of the phenotypic analysis on offer will both be key factors that will ultimately dictate the choice, scale and effect of future pharmaceutical–biotechnology company partnerships in this area.

It is both an exciting and a challenging time to be involved in establishing functional genomics strategy within the pharmaceutical industry. Recent advances in comparative molecular genetics and other techniques have heightened interest in the mouse as a means of identifying the genes underlying both monogenic and polygenic disease states[5]. Foremost among these is the prospect of gaining access to additional phenotypes from gene knockout mice on a previously unprecedented scale, thereby extending the portfolio of biological information used within a comparative, evidence-based target selection strategy. The challenge for individual corporate bodies will be balancing their strategic investments in mammalian gene knockouts against the other types of biological insight offered by the alternative functional genomic technology platforms.

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